RAPID METHODS FOR IDENTIFICATION OF LACTIC ACID BACTERIA AND THEIR APPLICATION TO FERMENTATION MONITORING

By

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This dissertation is dedicated to my father, Herman Clifford Freund. His constant support and interest in this enterprise inspired me to find the time in a busy work schedule to complete this work. I thank him for providing me with the means for the education necessary to obtain this highest degree. He has taught me how to ask questions, think independently, and have the confidence to follow my convictions.

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RAPID METHODS FOR IDENTIFICATION OF LACTIC ACID BACTERIA AND THEIR APPLICATION TO FERMENTATION MONITORING

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Lactic acid bacteria are the primary contributors to a variety of fermentations and may be problematic spoilage organisms However, the development of rapid methods for the identification and characterization of lactic acid bacteria has not kept pace with advancements of more clinically significant organisms In addition, the challange of effectively monitoring mixed strains has not been met

Three methods to characterize and identify lactic acid bacteria were examined and evaluated for their suitability for directly monitoring cultures within a food fermentation. The three methods were the Biolog biochemical test kit, which reflects an organism's phenotypic response to environmental substrates; the Microbial Identification System of fatty acid profiling which measures the organism's phenotypic structure; and the Qualicom Riboprinter(TM) method of ribotyping, which characterizes a portion of the bacterial genome using EcoRI restriction enzyme.

One hundred and thirty-one strains of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus* species were obtained from the American Type Culture Collection (ATCC) and throughout the food industry. When the ATCC strains were analyzed, ribotyping performed best with genus and species being identified correctly 82% and 75%, respectively. The Biolog correctly identified 72% and 53% and the fatty acid 71% and 25% respectively. For initial characterization of an isolate, a combination of methods was recommended to gain maximum information.

Finally, a method utilizing the riboprinter to directly monitor cultures in sausage and sauerkraut fermentations was

developed that provided results within 9 hours of sampling. Changes in the microbial population were successfully monitored and results were not affected by the presence of mixed cultures. The actual starter culture band patterns could be stored in a database for comparison to subsequent fermentations.

LITERATURE REVIEW AND INTRODUCTION TO THE RESEARCH

Lactic Acid Bacteria

Introduction

The lactic acid bacteria are Gram positive, catalase negative cocci or rods that do not produce spores They are generally non-motile and divide in one plane only These organisms are obligate carbohydrate fermenters with the main product of this fermentation being lactic acid Other volatile acids and carbon dioxide may also be produced (20,48,56)

The lactic acid bacterial group is comprised of the genera, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, as well as the lactic *Streptococcus* species (101,103 110) The group is usually divided into two subgroups based on their products of glucose metabolism homofermentative or heterofermentative (3,51) The homofermentative species ferment available sugars to produce lactic acid using the Embden Meyerhof pathway (also known as

glycolysis. This pathway is diagrammed in Figure 1 and is characterized by the formation of fructose 1,6-diphosphate which is cleaved by an aldolase into dihydroxyacetone phosphate and glyceraldehyde-3 phosphate. The glyceraldehyde 3-phosphate is then converted to pyruvate which, in turn, is reduced to lactic acid by a lactate dehydrogenase. This step reoxidizes the NADH that is formed during earlier steps. A redox balance is obtained and the primary end product is lactic acid (51). If pentoses are utilized rather than glucose, acetic acid or lactic acid may be produced in the case of homofermentative organisms. Under these conditions, they appear to be heterofermentative. Homofermentative organisms produce twice the amount of ATP compared to the heterofermentative group because they possess the enzymes aldolase and hexose phosphate isomerase (51). The heterofermentative species produce a lower quantity of lactic acid from hexose fermentation than homolactics, but produce carbon dioxide, ethanol and other volatile carboxylic acids in addition (9,101). They may also produce flavor and odor components such as acetaldehyde and diacetyl.

Heterofermentative organisms utilize the hexose monophosphate pathway (20,56). Figure 2 illustrates this

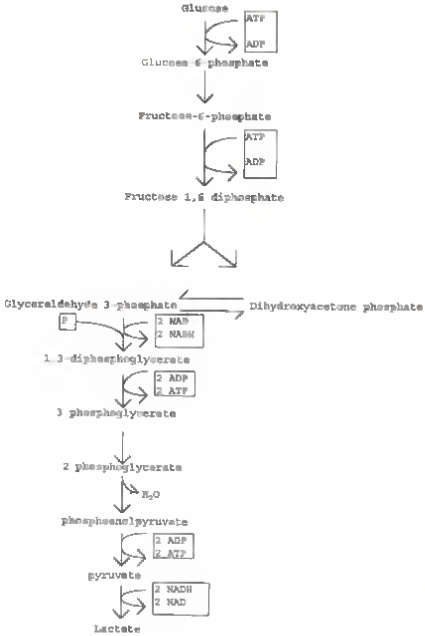


Figure 1 Embden-Meyerhof Pathway (51)

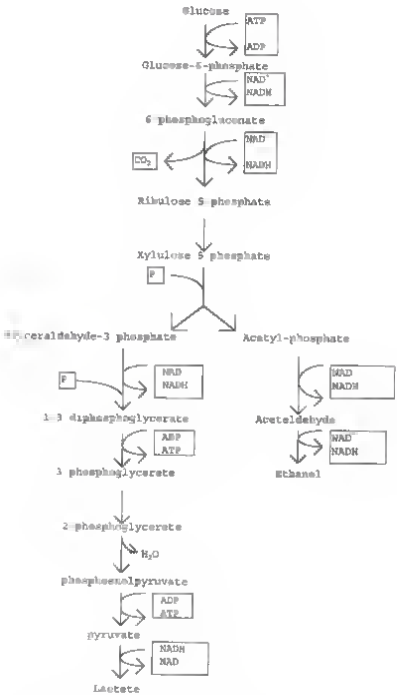


Figure 2 Hexose Monophosphate Pathyway (51)

pathway Since they lack aldolase, this pathway is less productive, yielding only one mole of ATP The first step is a dehydrogenation forming 6-phosphogluconate, which is followed by a decarboxylation Although heterofermentative organisms lack the aldolase, they do possess another enzyme, phosphoketolase Xylulose-5-phosphate is cleaved by the phosphoketolase to produce glyceraldehyde-3-phosphate and acetyl phosphate The glyceraldehyde is metabolized in the same way as the glycolytic pathway (Figure 1) which results in the production of lactic acid When pentoses are metabolized by heterofermentative organisms, no carbon dioxide is formed since no dehydrogenation step is necessary to reach the xylulose-5-phosphate intermediary Pentose fermentation yields lactic and acetic acid (51)

All of the *Pediococcus*, *Lactococcus*, and *Streptococcus* are homofermentative organisms The *Leuconostoc* are heterofermentative and the *Lactobacillus* may be either depending on the species (51,101)

Lactic acid bacteria were first classified in the early 1900's A group was created for bacteria that fermented milk causing coagulation (20,101). Orla Jensen in 1919 summarized, "The true lactic acid bacteria form a great

natural group of immotile, sporsless, gram-positive cocci and rods, which in fermenting sugar form chiefly lactic acid" (76,2)

The lactic acid bacteria are part of the normal flora of the mouth, lower intestine, and the vagina They are symbiotic and are generally a benefit to the host by stopping the invasion of other organisms that may not be beneficial (91,97)

Role in the Food Industry

Fermented Foods

Lactic acid bacteria are responsible for the fermentation of a variety of foods Fermentation is a process whereby carbohydrates or other compounds are oxidized to produce energy without the use of external electron acceptors (51) Fermented foods are important to man for a number of reasons Fermentation often increases the shelf-life of foods Also, it can increase the vitamin content, lend favorable flavor attributes, and increase the digestibility of the food Foods converted by microbial activity add variety to the diet because fermentation by-

products change the flavor and texture of the original food (51,74,95,97) In addition, in recent years, there has been an increased emphasis on nutrition which has led to a surge of interest in probiotic foods and natural preservatives (46,90) Benefits attributed to foods fermented with lactic acid bacteria include anticarcinogenic effects, lowering of cholesterol, and stimulation of the immune system (45,83,90)

Food Safety/Preservation

The lactic acid bacteria assist in food preservation by typically producing large quantities of lactic acid which creates a low pH environment that is not hospitable to pathogenic organisms as well as many other spoilage bacteria Lactic acid bacterial fermentations often result in an external pH of less than four The lactic acid not only inhibits other bacteria via pH depression, but metabolically inhibits them due to the undissociated acid molecules and, as pH decreases the inhibitory activity of the organic acids increases (6,9,26,46,74,101,103)

Fermented milk products do not have as significant a drop in pH due to the buffering capacity of the milk, yet

competitive microflora are still inhibited (73) The lactic acid bacteria have also been found to produce a number of antibiotic compounds. These compounds are called bacteriocins (74) They are heat resistant peptides or proteins whose genetic information is usually plasmid borne One such compound, nisin, has received much attention in recent years Nisin is a polypeptide containing 34 amino acids produced by *Lactococcus lactis* Like most bacteriocins, it is non-toxic to humans, produced naturally has good storage stability, and, yet, is destroyed by digestive enzymes (51 74) The Food and Drug Administration has granted GRAS (Generally Recognized As Safe) status to nisin (74) It does not contribute an off odor to the foodstuff to which it is added and has a narrow spectrum of antimicrobial activity It is effective against Gram positive organisms such as *Clostridium botulinum* and *Listeria monocytogenes* (29). Other bacteriocins include diplococcin and lactostrepcins (produced by species of *Lactococcus*), pediocins (produced by *Pediococcus* species), and a host of others, such as acidophilin, lactolin, and lactocin (produced by members of the lactobacilli) (74 103). Lactic acid bacteria used to produce fermented foods enhance

the safety of those foods by preventing the growth of food-borne pathogens as well as increasing the shelf life by inhibiting spoilage organisms (63,73)

The production of bacterocins hydrogen peroxide, organic acids, depressed pH, and nutrient depletion all contribute to the mechanics of lactic acid bacterial antagonism (1,21,25,26,29,63,101,106,113)

Use as Starter Cultures

Natural fermentations are those fermentations that are initiated without the addition of starter cultures Control of natural fermentations is achieved by altering the environment Natural fermentations are generally slower, but often yield more complex flavor components than in a controlled fermentation (74)

Controlled fermentations add starter cultures to raw food The use of starter cultures provides many advantages over the more traditional method of "natural" fermentation Starter cultures can be added in a known quantity in pure culture or by "backslopping " Backslopping is a method using a sample from a previous fermentation to inoculate fresh raw materials in an attempt to initiate a new

fermentation (74) The addition of known starter cultures is generally employed in industrial fermentations because it reduces processing time and decreases batch to batch variation which results in a more controllable fermentation Also, fermentation failures are less likely to occur because starter organisms are added in large quantities to ensure their success (95)

The ability to produce lactic acid and other flavor components makes the lactic acid group of organisms useful as starter cultures They aid in the fermentation of milk to produce acidophilus milk, buttermilk, cheese, kefir, and yogurt They are also important in the fermentation of meat to produce sausages, cabbage to produce sauerkraut, cucumbers to produce pickles, olive fermentations, sourdough bread flavor development, and have even been used in the wine industry to deacidify wines by converting malic acid to lactic acid and carbon dioxide (9,51,89,97,105).

In spite of nutritional fastidiousness, the lactic acid bacterial group has demonstrated growth in a variety of habitats that include neutral pH (7.0), neutral temperatures (25°C) low pH (3 0) low temperatures (5°C), and high salt concentrations (3%) (20 75) In the fermentation of

sausages starter cultures are used to produce cured or ripened sausages within 24 to 48 hours as opposed to traditional curing which can take up to 14 days. Sausage production was probably one of the first forms instances where fermentation was used as a method of food preservation The bacteria in sausage production serve to produce lactic acid from the fermentation of sugars and the resulting acidification reduces the drying time because the acid denatures the meat protein which aids in the expulsion of water (85,95) The competitive microflora present in raw meat is extremely high and sausage starter cultures must be fast growing and good competitors (62) Some members of the lactic acid bacterial group make good competitors not only because of their ability to grow quickly in a specific niche, but also because they are able to inhibit the competition through the production of their antimetabolites and bacteriocins (63) Lactic acid bacteria are also used in the cheese industry to ferment milk The starter culture is typically added to warm milk The lactose present in the milk is converted to lactic acid and small amounts of carbon dioxide, acetic acid, acetaldehyde, acetoin, and diacetyl These products contribute to the distinctive flavor that a

[illegible] flavor development also occurs during [illegible] cheese maturation In this stage, the curd is broken down due to proteolysis, lipolysis and other enzymatic processes which create texture and flavor differences (75) For most cheese types, during the first few weeks of maturation, the starter population rapidly declines but enzymes survive to continue the maturation process (i e , proteases and lipases) (20) An exception to this is cheddar cheese where the lactic streptococci and the lactobacilli increase in population until a maximum is reached, usually at 3 to 6 months (85) Although extensively studied, the dynamics of these mixed starter populations and their specific contributions to flavor and texture are not well understood (29)

Another industry that involves a lactic acid bacterial fermentation is the production of sauerkraut Sauerkraut is a natural fermentation of cabbage The external environment is altered by placing the cabbage in a salt brine in a similar manner as pickles and olives Salt is added to the raw cabbage and it extracts water from the cabbage thus forming the brine A succession of bacteria is involved in the fermentation It begins with *Leuconostoc mesenteroides*

and is followed by either *Lactobacillus brevis* or *Lactobacillus plantarum* and often, *Pediococcus* species. Since sauerkraut is a natural fermentation, there is the opportunity for the growth of undesirable microorganisms which can result in an inferior or unacceptable product. Researchers write that in order to achieve controlled regulation of the fermentation,

> it is necessary to gain more information on the role and limitations of the raw product, to define more closely those conditions which contribute to undesirable or abnormal end-product formation; and to establish conditions which are optimal for the growth of selective cultures. This knowledge when properly applied should offer a fermentation which can be controlled as desired. (20:279)

This sentiment is expressed by many industrial food microbiologists who rely on fermentations to produce their product (28).

Fermentations generally contain mixed populations of organisms, whether it be the starter culture and the normal flora of the raw materials, or a mixed starter population. The dynamics of mixed populations are a result of competition, growth stimulation, changes in redox potential, water activity, and pH, substrate changes; and the production of inhibitory substances or secondary metabolites (48). The interaction between two microorganisms and the

interaction between a microorganism and its environment are very important. Microbial growth may deplete certain nutrients whose absence could strongly affect another population. Changes in substrates could increase or decrease the ability of certain organisms to grow. An example of this is in the case of *Staphylococcus*. *Staphylococcus* is a poor competitor, but an increased salt concentration or a lowered water activity may decrease competitive microflora and provide *Staphylococcus* the opportunity to thrive. Byproducts of microbial growth may also affect members of a population. Examples of substances of microbial origin which are able to affect the growth of other microbes include: hydrogen ions, carbon dioxide, organic acids, peroxides, and antibiotics or bacteriocins (25,47,63,105).

Enzymatic reactions associated with microbial growth may result in the production of acid or alkaline conditions in foods. Acids, such as lactic acid, are produced mainly by the fermentation of carbohydrates. Alkaline conditions are achieved by the production of ammonia or amines during the breakdown of proteins. pH is a critical factor and slight changes can greatly influence the development of

microflora In natural fermentations acid production can select for acid tolerant organisms such as the lactic acid bacteria In starter cultures growth stimulating activities have been observed between yeasts, micrococci, streptococci, lactobacilli, bacilli, and the Enterobacteriaceae In this phenomenon, metabolic by-products of one microbe can be readily absorbed and utilized by others In the fermentation of olives it is believed that yeasts provide B vitamins that are necessary for the growth of *Lactobacillus plantarum* and recently it was discovered that two strains of *Lactobacillus* exist in such close association in sourdough cultures that they are impossible to culture individually (48,89,112)

These close associations of microorganisms lead to the development of symbiotic relationships Symbiotic relationships are those where two or more species exhibit maximum growth potential in the presence of each other The close association of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt cultures is one such example These organisms display greater metabolic activity in mixed culture than the sum of the two acting individually In the presence of *L bulgaricus*, *S*

thermophilus grows faster, reaches higher numbers, and as a result, produces more acid than it does in pure culture (48). These interactions are difficult to study due to the laborious process required to adequately monitor mixed cultures. In the case of the lactic acid bacteria, their meticulous growth requirements further complicate the issue.

Role as Spoilage Organisms

Although lactic acid bacteria are used as starter cultures, they may also be problematic spoilage organisms if they enter the food system uninvited.

Lactic acid bacteria show a high degree of adaptation to their environment. In spite of their highly fastidious nutritional requirements, they are still able to use relatively scarce energy sources, partly by the use of their oxidation reduction systems. As discussed previously, their nutritional preferences allow them to develop symbiotic relationships that enhance their survival and, at the same time, increase their difficulty to control.

In vacuum packaged meats, lactic acid bacteria predominate and are responsible for the formation of diacetyl which is often a component of the volatile

compounds in these meats To the consumer this is noticed as an off odor Lactic acid bacteria may also produce a ropy slime on the surface of the meat as well as causing greenness and souring (54). The lowered redox potential and increased carbon dioxide in vacuum packed meats favors the presence of lactic acid bacteria (71) The production of hydrogen peroxide by lactic spoilage organisms in the presence of oxygen causes an oxidation reaction with nitrosohaemochrome which results in the green discoloration that is unpleasant to consumers If nitrates are present, lactics will thrive because they are not sensitive to nitrites (102)

In the orange juice industry lactic acid bacteria such as *Leuconostoc* produce a buttermilk aroma which is extremely undesirable in this type of product (78 79) Lactics have been associated with the industry spoilage of salad dressings due to excessive gas production which resulted in "champagne" salad dressings (65) *Lactobacillus fructovorans* has been commonly associated with the spoilage of vinegar preserves thus providing an example of the ability of these organisms to withstand high levels of organic acids, in this case acetic acid (23) The lactic

group is also associated with the spoilage of beer and wine (2). In addition, non-starter lactics have been demonstrated to enter fermentation systems and produce biofilms in fermentation vats (96).

Fermentation Controls

Fermentation controls traditionally rely on creating the optimum environment for the growth of the desired organisms and the development of optimal starter strains. The strength of the starter culture is critical to successful fermentation technology. Starter culture organisms must be good competitors and fast growing. Often, starter strains are rotated to avoid phage infection. Good Manufacturing Practices (GMPs) and good sanitation practices are essential to ensure that the level of competitive microflora does not overcome the starter culture (78). The composition of the raw materials, the type of heat pretreatment, and incubation conditions are all factors that can be manipulated to ensure and maintain the artificial dominance of starter organisms over the natural flora (74). Once the fermentation has been initiated, there are a number of ways that it can be monitored.

Indirect Monitoring

Current controls for monitoring fermentations mainly rely on measuring the physical attributes of the food that is being fermented. In the meat industry, instrumental controls to continually measure aspects such as pH, water activity, and weight loss are used. In addition, other fermentation parameters may be controlled to ensure a safe product. For example, the American Meat Institute states that Good Manufacturing Practices for sausages include reaching a pH of 5.3 or less within 30 hours at 24°C. This is to ensure that pathogenic microorganisms such as *Staphylococcus aureus* are not given the chance to reach toxic levels prior to the pH drop where their growth is inhibited (42,62,82). Little information about the specific biochemical contributions of the starter cultures on flavor and aroma is known (62). This is one reason that the maintenance of the desired starter population throughout the fermentation is important.

Traditionally, mixed strain starter cultures have been used on a rotation basis to avoid phage infection and maintain a healthy starter population to control the outcome of fermentations. Other benefits of mixed strains are as

follows: activity may be less affected by changes in raw material composition; a lower inoculum level is needed; a more consistent end product is achieved, and the starter activity is more uniform (9). However, recent reports have suggested that there may be advantages to using a single strain. Continuous cultures could be applied and this reduction in bacterial diversity may lead to decreased phage diversity within manufacturing facilities (34). In this situation, the integrity of the single strain starter becomes even more critical and must be highly monitored to prevent failure.

Biosensors are self-contained analytical systems that are being developed for clinical use as rapid "on the spot" testing at the bedside. As their development continues, they may be expanded for use in continuous monitoring of fermentations. Currently, their application to food systems is hampered by difficulties such as electrode fouling, pH dependence, and non-uniform mixing of the substrate concentration as well as the presence of high concentrations of analytes (70).

Direct Monitoring

Direct monitoring of fermentation cultures has not been typically applied This is mainly due to the fact that in a production situation where large inoculums of quickly growing starter organisms are used, there is simply not enough time to run traditional microbiological tests to determine the types of organisms present in the culture

Recent concerns about emerging pathogens in products such as the presence of *E coli* O157:H7 in sausage highlight the need for quick and reliable means to directly monitor fermentations One challenge of direct monitoring is the rapid isolation of bacteria from the material undergoing the fermentation reaction Traditional plating methods do not provide timely results

Methods have been developed to extract the DNA of non-culturable organisms directly from soils and sediments These procedures fall into two categories, cells are lysed after removal from the sediment or cells are lysed within the sediment (58) Disruption of the cells within the sediment involves treatment of the sediment with lysozyme and rapid freeze-thaw followed by a phenol-chloroform extraction and precipitation of the DNA Finally,

[illegible] removed using gel filtration. The other [illegible] includes the use of glass beads to disrupt cells that [illegible] removed from sediment and centrifugation to remove the beads and sediment particles. This is followed by a phenol chloroform extraction and ultrafiltration. The lysis of cells after removal from the sediment provides the highest yield of DNA, but it is more fragmented due to shearing (58,119). Similar methods have been developed for DNA extraction of marine bacteria (60). These methods provide an advantage in that they have the ability to detect non-culturable organisms and they do directly extract DNA; however this extraction of the DNA is not timely. Most industrial fermentations will have finished prior to the analysis of the first sample. Also, the DNA, once extracted is often fragmented during the rigorous attempts to separate it from the sediment. One group of researchers (108) attempted a PCR amplification of DNA extracted from sediment, but discovered that amplification was inhibited. An electrophoresis step was necessary prior to performing PCR.

Culturing of Lactic Acid Bacteria

Lactic acid bacteria are difficult to grow in culture and, often, when they do grow, it is slow. They have fastidious nutritional requirements and require preformed amino acids, B vitamins, and purine and pyrimidine bases. These requirements often restrict them to environments where carbohydrate, protein breakdown products, vitamins and minerals occur in sufficient quantity and proportion to support their growth (31,51,77,110). This fastidious nature is believed to be caused by mutational losses of metabolic steps. It has been hypothesized that this is done by the organisms in an attempt to eliminate nonessential pathways to conserve energy by limiting metabolites (8). Researchers have developed a large number of broth and agar plating media in an attempt to optimize the growth of the more fastidious lactic acid bacteria (see Table 1). This is indicative of the difficulties encountered in growing members of this group. Media selection is controlled to some extent by the strains under study as well as the foodstuff from which they are isolated (4,31,48,98,101,110). The lactic acid bacteria are tolerant of low pH, but can be sensitive to other adverse conditions such as freezing

Table 1 Media for culturing lactic acid bacteria (3)

Media Name	Target Organism(s)
Leuconostoc oenos Medium	*Leuconostoc* oenos
M16, M17 Agar and Broth	Cheese starters/lactic strepa
MRS Agar and Broth	Lactic acid bacteria
Orange Serum Agar and Broth	*Lactobacilli*, acidburic organisms
Raka-Ray Agar	Lactic acid bacteria in beer
Sour Dough Medium	*Lactobacillus sanfranclaco*
Universal Beer Agar	Spoilage organisms in beer
Yeast Extract Glucose Citrate Media	*Leuconostoc* species
YGC with Cysteine	*Leuconostoc mesenteroides*
Yeast Extract Nutrient Gelatin	*Lactobacillus acidophilus*
Tomato Juice Agar Broth, Special	Lactic acid bacteria
Tomato Juice Yeast Extract Milk	*Lactobacillus, Enterococcus*
Micro Assay Culture Agar	Lactobacilli
Litmus Lactose w/crystal violet	Lactic acid bacteria
APT Agar and Broth	Heterofermentative *lactobacilli*
Glucose Yeast Extract Agar	*Leuconostoc, Pediococcus*
Lactic Agar for Yogurt Bacteria	*Lactobacillus species*
Lactic Acid Bacteria Medium	Lactic acid bacteria from wine
Lactobacillus Media	*Lactobacillus* species
HYA Agar	*Lactobacillus bulgaricus*
HF Medium	*Leuconostoc* species
Glucose Yeast Broth w/NaCl	*Pediococcus halophilus*
Elek Agar	Lactic streptococci
Double Strength for *Lactobacillus*	*Lactobacillus* species
Chopped Meat Media w/Tween 80	*Lactobacillus* species
Sarcobactarium Medium	*Lactobacillus* species
Lactobacillus Selection Oxgall Agar	Bile-resistant Lactobacilli
Lactobacillus Haters Screen Agar	*Lactobacillus* spp in salad dressing
Lactobacillus bulgaricus Agar	*Lactobacillus bulgaricus*
Acetate Agar	*Leuconostoc, Pediococcus*
Acidic Tomato Medium	*Leuconostoc* species
Agar for differential enumeration of Lactic Streptococci	Differentiation between *Lactobacillus lactis* subspp

Sterile peptone water is recommended for the dilution of samples because phosphate buffered diluent has been shown to damage the bacteria in samples to the point that reduced counts are obtained (48,98,110).

Since the lactic acid bacteria seem to demonstrate better growth in a reduced oxygen atmosphere, it is a common practice to incubate cultures under semi-anaerobic conditions in the presence of substantial concentrations of carbon dioxide (20,48,101,110)

There is no single medium that has been developed for the cultivation of all lactic acid bacteria. DeMan, Rogosa, and Sharpe (MRS) agar is the closest thing to a universal medium for the lactic acid bacterial group, but many isolates fail to grow on MRS (77). Lactic acid bacteria prefer fructose as a carbon source rather than glucose however, much of the commercially available media, including MRS, contains glucose and not fructose (48,65,101)

Classification/Taxonomy of Lactic Acid Bacteria

As microbiologists continue to explore new horizons and isolation methods become more advanced, it is apparent that there is a great degree of bacterial diversity (44)

Traditional classification of microorganisms has been based on biochemical and morphological similarities and differences. There is some concern that current taxonomy does not reflect the evolutionary origin of microorganisms (57-59). In the lactic acid bacterial group alone there exists a physiologically and morphologically diverse population (101). Classification of lactic acid bacteria has been approached using a number of methods including GC% ratio, DNA hybridization, serology, electrophoretic mobility of lactic dehydrogenases, biochemical, and fatty acid composition as well as simple gram reactions (27,28,30,32,37,51,86,93,100,107).

The GC ratio varies from a ratio of 53 for *Lactobacillus fermentum* to 35 for *Lactobacillus salivarius*. When organisms are grouped according to their GC ratios, homofermenters and heterofermenters overlap the groups thus emphasizing the diversity (51).

Many automated identification systems exist for the identification of clinical specimens, but there are few systems developed that can identify environmental isolates quickly and reliably. Test kits for non-enteric organisms are not common and are often labor intensive and slow (53).

The basic premise of strain typing or differentiation is that clonal descendants from a single ancestor will possess characteristics that are different from descendents of a different ancestor Strain typing has been used historically for epidemiological purposes in the medical industry to determine whether origins of infections were related Due to technology constraints, traditional methods of strain typing use phenotypic expressions such as serology, biotyping and morphology The lactic acid bacterial group is extremely diverse and the application of phenotypic methods is difficult Antibiotic resistance has proven successful for the classification of this group and it has been used in both the food and medical industries However, in recent years more sophisticated methods of strain typing have emerged These include the use of molecular techniques which, theoretically, should be able to specifically determine clonality The development of these techniques specific to the lactic acid bacterial group has not kept pace with the common pathogenic organisms due to the lactic group s limited role in clinical epidemics Plasmid profiling is a popular method applied to the lactic acid bacteria but unfortunately, this is not a stable

molecular identification technique due to the fact that bacterial strains may gain or lose plasmids

The use of patterns generated from the digestion of chromosomal DNA using restriction endonucleases is sensitive and may have the potential to differentiate between similar strains. Ribotyping, which types strains based on patterns generated by the ribosomal RNA genes, has been successful for distinguishing members of this group (19,32,59) In some instances ribotyping has not been discriminatory enough and the application of pulsed field gel electrophoresis has been more successful (32,94) It should be noted, however, that molecular techniques tend to be more time consuming and expensive than simple plasmid profiling or serology. In addition there are few studies on the lactic acid bacteria to demonstrate the degree of reproducibility of the techniques Only about one-third of the species of lactic acid bacteria have been extensively studied Most studies have concentrated on *Lactobacillus helveticus*, *L acidophilus* *L fermentum*, *L plantarum*, *L casei*, *L reuteri*, *L sake*, and *L curvatus*. Many studies have investigated the gastrointestinal flora of animals to give insight into the role these organisms play in the intestinal ecosystem In the food industry, the persistence of

Lactobacillus starter cultures has been monitored in silage and to measure diversity in natural fermentations such as olives, fermented meats, and sourdough (32,59,112) There has been limited application to continuous monitoring of starter cultures or attempts to trace spoilage due to lactic acid bacteria within a food processing environment.

Since strain typing of the lactic acid bacteria has not kept pace with advances of more medically "important" bacteria, there exists a need to do more comparative research between techniques and the possible development of typing systems for the more important lactobacilli. The application of typing techniques to a wider range of species and environments as well as studies tracing the sources of contaminating organisms are required. "The importance of the genus, *Lactobacillus*, in both the industrial sphere and human and animal health warrant the extensive development and implementation of strain typing techniques for this genus" (35 65)

Rapid Methods of Identification

The use of rapid identification methods in the field of microbiology has grown in recent years. The importance of quick diagnosis in the medical industry is obvious. In the food industry, the benefits to rapid analysis are tremendous. Cost savings can be achieved in terms of raw material and finished product storage alone. If positives are detected, product can be widely implicated and down time of equipment during cleaning may be extensive. Traditional biochemical culturing and classification is still useful and necessary, but even those tests are becoming automated. The introduction of computers has significantly advanced these rapid identification methods as reaction patterns or restriction patterns generated can be stored and compared to relatively large databases.

Rapid methods have been developed to simplify culturing techniques such as automatic diluters, platers, and the development of membrane filters and traditional agar replacements. Enzyme linked immunosorbant assays (ELISA) DNA probes and Polymerase Chain Reaction (PCR) technology have all emerged to speed the process of bacterial culturing and identification (39).

In this research, three methods to characterize and identify lactic acid bacteria were tested. The methods include a biochemical one, which reflects an organism's phenotypic response to environmental substrates, fatty acid profiling, which measures the organisms phenotypic structure; and ribotyping, which characterizes a portion of the bacterial genome. The challenge to using standard, developed methods is that they often will only successfully identify a small percentage of environmental isolates (18). Many of the rapid tests for foodborne bacteria are adaptations of ones developed for clinical isolates, but the background microflora is much different for food as well as the level of acceptable organisms (117). In the food industry few tests specific to foodborne bacteria exist and clinical databases or ATCC databases often do not provide data for the reliable identification of environmental food isolates. The advantages of these systems are the speed of the analysis and the high sensitivity when compared to classical methodology. Also, results can be stored in computer databases and used for future comparisons which is extremely important in epidemiological considerations.

Biolog Automated Microbial Identification System

Biolog (Hayward, CA) was developed in 1989 as a method for identifying aerobic Gram negative bacteria such as the enteric bacilli, non-fermenters, and some fastidious species. It is an automated system equipped with a computer and the appropriate software, a turbidimeter, a microplate reader and coated microwell plates (11). Currently, kits are available for a broad range of bacteria including cocci, bacilli, and spore formers. Identification is made by determining the carbon source utilization profile of a particular strain. The kit consists of ninety-six dehydrated panels that contain tetrazolium violet, a buffered nutrient medium, and a different carbon source for each well, except the control well. Table 2 lists the carbon sources for the wells.

The indicator is tetrazolium violet, a redox dye, which is used to detect electrons donated by NADH to the electron transport system. When reduced, tetrazolium violet is a purple formazan; therefore, when a carbon source is not used, the microwell remains colorless. A pattern of purple and colorless wells is created that yields a metabolic fingerprint for each organism tested (55). The ninety-five

Table 2 Biochemical sources for Biolog lactic acid plate (11)

α-cyclodextrin	β-cyclodextrin	dextrin	glycogen
inulin	mannan	Tween 40	Tween 80
N acetyl D-glucosamine	N-acetyl-D-mannosamine	D-galacturonic acid	mono-methyl succinate
D arabitol	erbutin	cellobiose	D-fructose
L-fucose	D-galactose	amygdalin	gentiobiose
D-glucosaic acid	α-D-glucose	α lactitol	α-D lactose
lactulose	maltose	maltotriose	D mannitol
D mannose	D-melezitose	D-melibiose	3-methyl glucose
β methyl D galactoside	α-methyl-D-galactoside	α methyl-D-glucoside	β methyl D glucoside
acetic acid	palatinose	D-psicose	D raffinose
L-rhamnose	D-ribose	salicin	sedoheptulosan
D sorbitol	stachyose	sucrose	D-tagatose
α trehalose	turanose	xylitol	D xylose
α methyl D-mannoside	α hydroxybutyric acid	β-hydroxybutyric acid	G-hydroxybutyric acid
p hydroxyphenyl acetic acid	α-ketoglutaric acid	α ketovaleric acid	N-acetyl L glutamic acid
methyl pyruvate	L-lactic acid	D-malic acid	L malic acid
L arabinose	L serine	propionic acid	pyruvic acid
succinamic acid	succinic acid	bromamide	alaninamide
D-alanine	L alanyl glycine	L asparagine	L-glutamic acid
glycl L glutamic acid	L-pyroglutamic acid	D lactic acid methyl ester	2 deoxy adenosine
2 3 butanediol	glycerol	adenosine	putrescine
inosine	thymidine	uridine	
tymidine 5 monophosphate	uridine 5 monophosphate	fructose 6 - phosphate	glucose 1 phosphate
glucose 6 phosphate	D-L α-glycerol phosphate	adenosine 5 - monophosphate	

tests are able to provide identification for fifty seven strains of lactic acid bacteria. These strains are listed on Table 3. Once the strain of interest is cultured on a solid agar medium, the cells are removed from the surface and suspended in a liquid medium to a specified optical density. The 96 microwell plate is inoculated with the cell suspension and incubated. Reactions may be read in 4 or 24 hours and an identification is made. One publication (53) accepted identifications of Gram negative bacteria if the similarity indices were 0.500 or higher at 24 hours. Of the 41 isolates run in their study, 40 (98%) were identified to the correct genus and 24 (59%) were identified to the correct species. These researchers found that incubating Gram negative plates for 6 to 8 hours and using the 4 hour database resulted in optimal performance although it was not recommended by the manufacturer (53).

Biolog identifications are performed using numerical taxonomy. The well reactions are coded as binary variables (i.e. positive or negative) for each individual test or well. Once the classification is achieved, a percentage positive table is generated that shows the percent of strains within the group that tested positive for each well.

Table 3. Database for the Biolog lactic acid bacteria (11)

Lactobacillus acidophilus	*Lactobacillus plantarum*
Lactobacillus alimentarius	*Lactobacillus reuteri*
Lactobacillus animalis	*L. salivarius ss salicinius*
Lactobacillus bifermentans	*L. salivarius ss salivarius*
Lactobacillus brevis	*Lactobacillus vaginalis*
Lactobacillus buchneri	*Lactobacillus viridescens*
Lactobacillus casei ss casei	*Pediococcus acidilactici*
Lactobacillus casei ss rhamnosus	*Pediococcus dextrinicus*
Lactobacillus curvatus	*Pediococcus pentosaceus*
L. coryniformis ss coryniformis	*Pediococcus urinaequi*
Lactobacillus curvatus	*Leuconostoc carnosum*
Lactobacillus delbrueckii ss lactis	*Leuconostoc citreum*
Lactobacillus farciminis	*Leuconostoc gelidium*
Lactobacillus fermentum	*Leuconostoc lactis*
Lactobacillus fructivorans	*L. mesenteroides ss cremoris*
Lactobacillus gasseri	*L. mesenteroides ss dextranicum*
Lactobacillus hilgardii	*L. mesenteroides ss mesenteroid*
Lactobacillus kefiranofaciens	*Leuconostoc oenos*
Lactobacillus helveticus	*Leuconostoc paramesenteroides*
Lactobacillus jensenii	*Leuconostoc pseudomesenteroides*
Lactobacillus kefir	*Lactococcus garvieae*
Lactobacillus malefermentans	*Lactococcus lactis ss cremoris*
Lactobacillus sakei	*Lactococcus lactis ss diacetylactis*
Lactobacillus minor	*Lactococcus lactis ss hordniae*
Lactobacillus murinus	*Lactococcus lactis ss lactis*
Lactobacillus oris	*Lactococcus plantarum*
Lactobacillus parabuchneri	*Lactococcus raffinolactis*
L. paracasei ss paracasei	*Tetragenococcus halophilus*
Lactobacillus pentosus	

These values are used to generate an identification matrix which selects a minimum number of criteria deemed to be discriminatory for the classification group This calculation is then verified by practical trials (18)

Analysis of Cellular Fatty Acids

A large number of fatty acids are found in the cell wall and cell membranes of bacteria The composition of these cellular fatty acids is a genetic trait that is highly conserved within a taxonomic group With controlled culture conditions a cellular fatty acid profile is a very stable phenotypic expression of a bacterial genotype Fatty acid profiles are not prone to alteration by moderate genetic mutations and closely related bacteria have very similar fatty acid profiles Thus cluster analysis of the cellular fatty acid profiles can be used to group bacteria Analysis of cellular fatty acids uses actual cellular material in contrast to traditional biochemical methods that measure cellular density or color changes due to pH (39)

The procedure for analysis of cellular fatty acids involves the isolation and resuscitation of the original strain, cultivation hydrolysis of fat, fatty acid

derivatization, GC analysis of the fatty acid methyl esters, and, finally, comparison of these results to a database. The most important of these steps is the cell treatment during resuscitation and cultivation. Factors such as temperature, pH, salt content of the medium, and growth stage at the time of harvesting can all affect the final profile, which, in turn affects the comparison to a database (40). For cellular fatty acid analysis of lactic acid bacteria, a relatively large amount of bacterial cells must be harvested from solid plating media. This growth must be in pure culture. Standardized growth conditions are essential in order to obtain profiles for comparison. This is due to the fact that the cell envelope responds to environmental changes and cells alter the fatty acid composition of their lipids to maintain fluidity. Phospholipids form the bilayer of this membrane and this structure is composed primarily of straight chain fatty acid esters linked to glycerol phosphates. The time and temperature of incubation as well as the growth media must be established prior to making fatty acid composition comparisons. For example, as incubation temperatures decrease, cells adjust by increasing their percentage of

unsaturated fatty acids to maintain fluidity Lower chain length and increased double bonds will also provide this effect (61,68) Colonies should be harvested in the late log phase because this is the phase that yields the most stable fatty acid compositions However, they cannot be harvested too late in the log phase In *Vibrio* cells, the percentage of saturated fatty acids increased from 49 to 69 5% and the unsaturated species decreased from 45 to 17% as the cells aged and previously undetected long chain fatty acids appeared (61) A similar trend was observed with *E coli* cells (61) If cell culture conditions are rigidly standardized the fatty acid composition should be consistent

After harvesting, whole cells of bacteria or yeasts are saponified in a strong base (sodium hydroxide-methanol at 100°C for 30 minutes) to release the cellular fatty acids from cellular lipids, the second step is methylation with HCl in methanol at 80°C for 10 minutes The fatty acid methyl esters are extracted into a mixture of hexane and methyl tertiary butyl ether for 10 minutes and, finally the extract is washed in aqueous sodium hydroxide The fatty acids are converted to a methyl ester derivative to increase

volatility and then analyzed using gas chromatography (68) The system identifies dimethyl acetyls, aldehydes and other unknown compounds in addition to the fatty acid methyl esters (92,105)

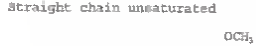
Cellular fatty acids are usually defined as those from 9 to 20 carbons in length which includes the majority of the fatty acids in the cell membrane in the form of glycolipid and phospholipid- Most bacteria synthesize fatty acids with a chain length of 10 to 19 The 16 to 18 length chains are the most predominant The acids are named according to the number of carbons in the chain, the type of functional group, and the double bond location Carbons are counted from the carboxyl end (92) Figure 3 shows some examples of fatty acids

Hexadecanoic acid, a highly conserved fatty acid within the prokaryotic organisms, would be denoted $C_{16:1}$ It includes constituents of the lipopolysaccharides (LPS), but not the very long chains found in mycolic acids In addition to the membrane fatty acids the Lipid A component of the LPS layer is a major source of fatty acids in the Gram negative bacteria In Gram positive organisms, it is the lipoteichoic acids Fungi produce sterols as the major lipid The type of fatty acid produced by a particular

Straight chain saturated

18:0 fatty acid 18:0 fatty acid methyl ester

Straight chain unsaturated

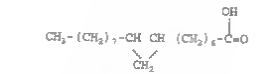


18:1 cis 9

Branched chain

Iso Anteiso

Cyclic



19:0 Cyclopropane

Hydroxy

alpha (2 OH) beta (3 OH)

Figure 3 Fatty acid structures (66)

organism is indicative of the biosynthetic pathway used to make that fatty acid The Coenzyme A ester of a fatty acid is synthesized using acetyl coenzyme A as the primer. More than one hundred fatty acids have been identified and the type of fatty acid as well as the quantity present in the cell is unique to that organism Theoretically, this means that differentiation between 2^{100} combinations are possible

Usually a range of 5 to 15 fatty acids are predominant in any one organism. The most common are saturated, straight-chain or monounsaturated acids with single double bonds in the chain Fatty acids with branched carbon chains are less common than straight chain and usually result in "iso" and "anteiso" acids (68) Gram negative bacteria tend to have a higher percentage of saturated and monounsaturated fatty acids with even numbered chain lengths The Gram positive group tends to have more branched chain, odd numbered chain lengths and lower levels of straight chain saturated fatty acids than the Gram negative (68) Gram positive organisms that do not possess the branched chain fatty acids are, therefore, more similar to the Gram negatives in this aspect These bacteria are the

[illegible]eforms and atraptococci which have atraight chained fatty acids and unsaturated fatty acids

Di- and tri-unsaturated fatty acids have been isolated from yeasts and the only common cyclic fatty acids are isolated from Gram negative bacteria (within the carbon chain is a 3 carbon cyclopropane ring) (68) Hydroxy fatty acids have an OH group While most fatty acids are completely saturated or lack a side chain functional group, occasionally fatty acids with more than one functional group are isolated Since they occur in so few organisms these acids are an immediate clue to the bacterial source (66) It was discovered early that avirulent strains of *Vibrio cholerae* did not have branched chain fatty acids but virulent strains did possess these fatty acids Similar discoveries were made with other organisms The fatty acid profile corresponds to the expression of nuclear genetic information and, therefore, cannot be influenced by plasmids (68)

The Microbial Identification System (MIDI) (Microbial ID Inc , Newark, DE) was used for this study The equipment involved in the analysis of cellular fatty acids includes a gas chromatograph with a flame ionization detector, a

silicone fused silica capillary column, an automatic sampler, integrator, computer, software, and a printer After the sample is injected into the column, the fatty acids are separated based on retention times under the conditions of increasing heat The carrier gas is hydrogen When the methyl ester of the fatty acid reaches the end of the column it is detected by the flame ionization detector and the signal is recorded as a peak The amount of fatty acid present is reflected in the area underneath the peak The size of the fatty acid is reflected by the retention time The amounts of cellular fatty acids are calculated as a percentage of the total fatty acids detected The use of fused-silica capillary columns as opposed to packed columns allows the recovery of hydroxy fatty acids and the ability to distinguish several isomers of fatty acids with the same length of carbon chain (66) The Microbial Identification System has the ability to analyze more than 300 fatty acid methyl esters ranging in length from 9 to 20 carbons The computer analyzes the chemical data using a multivariate statistical method of principal component analysis to interpret the data in order to give an identification Principle component analysis determines the most important

features in the chemical composition for distinguishing one species from another (66) Strains in the database are listed in Table 4 The software will generate a plot or a dendrogram to portray data about the samples (64). New library entries can be created for environmental isolates that may not match the database (12,93) If cells are cultured under alternate conditions, composition profiles can be reliably compared with profiles of other cells as long as they are cultured under those same alternate conditions (43)

The Microbial Identification System database includes information for the identification of species or subspecies for the genera *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, *Bacillus*, and 53 additional genera of aerobic bacteria In addition, it includes information for identification of mycobacteria, anaerobes, and yeasts The databases contain profiles of strains collected worldwide that have been grown under standardized conditions (66) Reports vary on the usefulness of this method for identifying to the genus and species level (12,40,41 61,68,91)

Table 4. Database for the MIDI fatty acid analysis (66)

Lactobacillus acidophilus	*Lactobacillus sanfrancisco*
Lactobacillus bifermentans	*Lactobacillus viridescens*
Lactobacillus brevis	*Pediococcus acidilactici*
Lactobacillus buchneri	*Pediococcus damnosus*
Lactobacillus casei	*Pediococcus dextrinicus*
Lactobacillus catenaformis	*Pediococcus halophilus*
Lactobacillus confusus	*Pediococcus parvulus*
L delbrueckii ss bulgaricus	*Pediococcus pentosaceus (MRSA)*
L delbrueckii ss delbrueckii	*Pediococcus pentosaceus (TSBA)*
Lactobacillus delbrueckii ss lactis	*Leuconostoc lactis*
Lactobacillus farciminus	*L mesenteroides ss cremoris*
L fermentum GC subgroup A & B	*L mesenteroides ss dextranicus*
Lactobacillus fructivorans	*L mesenteroides ss mesenteroides*
Lactobacillus helveticus	*Leuconostoc oenos*
Lactobacillus jensenii	*Leuconostoc paramesenteroides*
Lactobacillus minifermentans	*Leuconostoc pseudomesenteroides*
Lactobacillus reuteramicus	*Lactococcus garvieae*
L paracasei ss paracasei	*Lactococcus lactis ss cremoris*
Lactobacillus plantarum	*Lactococcus lactis ss hordniae*
Lactobacillus rhamnosus	*Lactococcus lactis ss lactis*
Lactobacillus ruminis	*Lactococcus plantarum*
L salivarius ss salicinius	*Lactococcus raffinolactis*
L salivarius ss salivarius	

Ribotyping

DNA fingerprinting has been used for epidemiological studies to compare organisms that were phenotypically indistinguishable. This method is based on the restriction fragment length polymorphisms in the DNA encoding for the ribosomal RNA (rRNA). The gene sequence coding for the 16S and 23S portions of the rRNA is a successful probe target site because it is universally distributed, there are many copies per cell, and it is highly conserved (13). Ribotyping band patterns reflect the number and distribution of the rRNA operons which gives an indication of the genomic organization and, therefore, species relatedness (18). Because ribotyping is not based on phenotypic expression it can offer a precise method for characterizing species and distinguishing strains (7). It has successfully differentiated between species and intraspecies. Ribosomal nucleic acids are considered "phylogenically meaningful" and are therefore used for evolutionary classifications. This molecular information is leading to the reinterpretation of many evolutionary relationships at all taxonomic levels (13).

Ribotyping has been applied to epidemiological situations. Environmental *Vibrio cholerae* strains in Thailand were grouped and characterized based on their ribotype band pattern (24). Methods that rely on phenotypic expression of the genome can be affected by stress placed on the organism. In the food industry, cells are often stressed by processing or drying (117).

The ribotyping method of identification requires the removal and isolation of the bacterial genome. This is accomplished by perforating the cell wall to release the DNA. After this extraction, the DNA is heated and then digested using a restriction enzyme. In this research the Dupont Riboprinter [TM] was used which applies the *EcoRI* restriction enzyme. The DNA fragments that are produced from this digestion are electrophoretically separated by size and transferred to a membrane where they are hybridized with a probe directed at the highly conserved DNA sequence the ribosomal DNA. They are then treated to provide a chemiluminescence reaction which is detected by an image capture system. The DNA pattern is compared to a database based on spatial relationship as well as the signal intensity to determine the relatedness of the species. The

image or DNA fingerprint, can be stored in the database and compared to future isolates or to image patterns in a recognition library (33). The database for the Riboprinter contained patterns for *Salmonella*, *Listeria*, *E. coli*, and *Staphylococcus*. It did not contain a database for the lactic acid bacterial group.

Data Analysis

All the methods described above depend on the estimation of the similarities between pairs of things. Cluster analysis is the most basic method for estimating similarities. In this study, the organism's attributes are measured using one of the three identification methods and the data is entered into a data matrix. The data is then standardized and a resemblance coefficient is used to measure the overall similarity between objects. These methods all use a similarity coefficient for the raw data which means that the larger the coefficient, the more similar the items being compared are. When the numbers are charted in a dendrogram, a dissimilarity coefficient called the Euclidean distance is used. The Euclidean distance measures the distance between two points when they are

measured in two dimensional space Euclidean distance is calculated using the Pythogorean theorum Values obtained are used to create a map or tree showing similarity called a dendrogram Fatty acid comparison relies on principal component analysis Cluster analysis is used to make taxonomic classifications The number of classes for taxonomic purposes is arbitrary and is a tradeoff between the level of detail within a class and the desire for generality or fewer classes Numerical taxonomists use cluster analysis by cutting the tree into clusters which become the archetypical classes of a classification Once classes are formed, unknowns can be identified and classified based on their fit into the class (88,93)

Introduction to the Research

Lactic acid bacteria are the primary contributors to a variety of fermentations yet this group of organisms have been studied relatively infrequently Fermentation often increases shelf-life, can increase vitamin content lend favorable attributes, as well as increase digestibility (51 94,96) The ability to control fermentations is one that has challenged the food industry In order to gain

more information on the role and limitations of the raw product; to define more closely those conditions which contribute undesirable or abnormal end-product information, and to establish conditions which are optimal for the growth of selective cultures. Current controls for monitoring fermentations rely on measuring physical attributes such as pH and water activity. The challenge of effectively monitoring mixed strains has not been met and therefore, direct monitoring of fermentation cultures has not been typically applied. In this research, three methods to characterize and identify lactic acid bacteria were examined and evaluated for their suitability to directly monitor cultures within a food fermentation. The three methods were the Biolog biochemical test kit, which reflects an organism's phenotypic response to environmental substrates; the Microbial Identification System of fatty acid profiling which measures the organism's phenotypic structure, and the Dupont Riboprinter method or ribotyping, which characterizes a portion of the bacterial genome using *EcoRI* restriction enzyme. The methods were analyzed for their ability to correctly identify ATCC isolates and industrial food isolates as well as their ability to distinguish between

strains Cluster analyses of spoilage organisms from different sources were performed in order to learn more about the lactic acid bacterial group Finally, a method to directly monitor mixed cultures in two food fermentations was developed

A comparison study to determine the optimal growth conditions and plating media for certain American Type Culture Collection (ATCC) strains of lactic acid bacteria was conducted

MATERIALS AND METHODS

Bacterial Cultures

Forty-nine strains of lactic acid bacteria, originating from the American Type Culture Collection (ATCC, Rockville, MD) and eighty-two strains isolated from the food industry were used in this investigation. A preliminary identification of the industrial isolates was performed using morphology and traditional methods of basic biochemical tests.

The strains were stored for long-term preservation at -80°C in preservation broth as shown in Table 5.

Table 5 Long term preservation media

Ingredient	Amount
MRS Broth	10 ml
Reconstituted Nonfat Dry Milk	10 ml
Glycerol	2 ml

The cultures to be preserved were grown in fresh MRS broth overnight at 25°C or until turbid growth was observed. The MRS broth with bacteria was mixed with presterilized, reconstituted, nonfat, dry milk and glycerol and placed in Corning disposable 2 ml polypropylene cryogenic vials (Fisher catalog number 03-374-6) and placed in deep freeze storage (-80°C)

Table 6 lists the ATCC strains used in this research. The ATCC designation is shown in parentheses and the reference number for this work is listed in Column 2. Table 7 lists the industrial isolates tested in these experiments.

Comparison of Plating Media

A comparison of plating media was performed using the lactic acid bacterial strains obtained from the American Type Culture Collection. The strains were resuscitated and transferred to 10 ml of sterile Trypticase Soy Broth (TSB) (Difco, Detroit, MI). The cultures were grown for 48 hours without shaking at 25°C. A Gram stain and catalase test was performed on each culture to verify the purity of the strain. At 48 hours an optical density (O.D.) at 590 nm using a Biolog Spectrophotometer Model 21101 was taken to

Table 6. ATCC strains used in this research

Organism Name (ATCC Number)	Number
Lactococcus garvieae (43921)	7340
Lactococcus lactis (29071)	7341
Lactobacillus acidophilus (4356)	7344
Lactobacillus alimentarius (29643)	7345
Lactobacillus amylovorus (33620)	7346
Lactobacillus animalis (35046)	7347
Lactobacillus bifermentans (35409)	7348
Lactobacillus brevis (27305)	7351
Lactobacillus brevis (14869)	7349
Lactobacillus casei ss casei (393)	7353
Lactobacillus casei ss rhamnosus (7469)	528
Lactobacillus coilinoides (27612)	7354
Lactobacillus confusus (10881)	7355
L coryniformis ss coryniformis (25602)	7356
L coryniformis ss torquens (25600)	7330
Lactobacillus curvatus (25601)	7332
Lactobacillus delbrueckii (9649)	7334
Lactobacillus delbrueckii (8000)	622
Lactobacillus farciminis (29644)	7335
Lactobacillus fermentum (14931)	7336
Lactobacillus fructosus (13162)	7338
Lactobacillus heintolerans (35410)	7358
Lactobacillus helgardii (8290)	7360
Lactobacillus malefermentans (49373)	7364

Table 6--continued

Lactobacillus minor (33412)	7380
Lactobacillus murinus (35020)	7387
Lactobacillus parabuchneri (49374)	7389
Lactobacillus paracasei (25599)	7371
Lactobacillus paracasei (25302)	7370
Lactobacillus pentosus (8041)	7372
Lactobacillus plantarum (14917)	7373
Lactobacillus plantarum (8014)	825
Lactobacillus reuteri (23272)	7374
Lactobacillus sake (15521)	7375
Lactobacillus salivarius (11742)	7376
Lactobacillus sumbicus (49375)	7378
Pediococcus acidilactici (33314)	4302
Pediococcus acidilactici (8081)	826
Pediococcus acidilactici (8042)	827
Pediococcus damosus (29358)	4303
Pediococcus dextranicus (33087)	4299
Pediococcus pentosaceus (33318)	712
Pediococcus urinaeequi (29723)	4300
Leuconostoc citreus (49370)	4301
Leuconostoc lactis (19258)	4297
Leuconostoc mesenteroides (19255)	4001
Leuconostoc mesenteroides (19254)	711
Leuconostoc paramesenteroides (3313)	588
Leuconostoc pseudomesenteroides (49371)	4298

Table 7 Bacteriai isolates obtained from industry

Isolate Source	Number	Isolate Source	Number
Dairy culture	KR5	Orange juice	UF20
Dairy culture	KR1	Orange juice	UF20
Dairy culture	KR309	Orange juice	UF21
Dairy culture	KR308	Orange juice	UF66
Dairy culture	KR141	Orange juice	UF83
Dairy culture	KR142	Orange juice	UF88
Dairy culture	KR137	Orange juice	UF98
Dairy culture	KR343	Orange juice	UF42
Dairy culture	KR17	Orange juice	UF15
Dairy culture	KR317	Orange juice	UF92
Dairy culture	KR18	Orange juice	UF68
Dairy culture	KR19	Orange juice	UF98
Dairy culture	KR22	Orange juice	UF65
Dairy culture	KR23	Unknown	CL119
Dairy culture	KR488	Unknown	CL124
Dairy culture	KR485	Unknown	CL102
Dairy culture	KR318	Unknown	CL103
Dairy culture	KR8	Unknown	CL105
Dairy culture	KR489	Unknown	CL108
Dairy culture	KR494	Beverage spoilage	CL109
Beer spoilage	ML6	ice cream slurry	CL123
Beer spoilage	ML7	Unknown	CL127
Beer spoilage	ML8	Unknown	CL126
Beer spoilage	ML5	Orange juice conc	CL122
Beer spoilage	ML2	Beverage spoilage	CL112
Beer spoilage	ML4	Beverage spoilage	CL113
Beer spoilage	ML1	Beverage spoilage	CL110
Orange juice	UF30	Unknown	CL100
Orange juice	UF71	Unknown	CL101
Orange juice	UF34	Unknown	CL104
Orange juice	UF18	Pet food spoilage	CL116
Orange juice	UF16	Unknown	CL121
Orange juice	UF10	Meat spoilage	OMLA2
Orange juice	UF33	Meat spoilage	OMLA7
Orange juice	UF27	Meat spoilage	OMLA8
Orange juice	UF72	Meat spoilage	OMLS7
Orange juice	UF	Meat spoilage	OM
Orange juice	UF26	Bakery	BAK10
Orange juice	UF17	Bakery	BAK18
Orange juice	UF16	Bakery	BAK14
Orange juice	UF24	Bakery	BAK5

determine the amount of growth Uninoculated TSB was used as a blank for the O D A cell density of 0 05 O D at 590 nm was targeted This O D was achieved by diluting the broth with sterile TSB The dilution was plated simultaneously on Trypticase Soy Agar (TSA, Difco) to determine the actual viable inoculum Five plating media were used for the comparison These included APT agar + 2% fructose, APT agar + 2% sucrose MRS agar (Oxoid, Unipath, Basingstoke, England), YM agar (Difco), and BLA agar (Biolog) Tables 8, 9, and 10 provide a list of ingredients for the different plating media

The plates were prepoured and the bacterial suspension was added to the surface using a sterile pipette and spread using a sterile glass hockey stick Four plates were inoculated for each organism All plates were incubated for 24 hours at 25°C Two plates were incubated aerobically and two in a CO_2 incubator Overlays were not attempted due to the requirement to harvest the cells for the three identification techniques

Cells were harvested by adding 5 ml of tryptone buffer to the agar surface and manually scraping this surface with a loop to remove the cells A sterile suction pipette was

used to suction off the broth now turbid with cells. The cells were placed into a sterile tube. The optical density was used to measure the number of cells to provide an indication of the amount of growth. Blanks were created for each type of plating media by adding 5 ml of sterile tryptone buffer to a sterile plate and using that liquid to calibrate the spectrophotometer.

Thirty strains were tested in the original experiment. The procedure was repeated two more times using 10 strains in duplicate and the three best performing media from the first experiment, MRS, MLA and APT + 2% fructose. The level of inoculum was reduced to an O.D. of 0.01.

Comparison of Identification Methods

The total amount of growth, measured in absorbance, for each of the strains tested on each medium was added together to achieve a total, combined absorbance for each plating medium. The means were compared using a One Way Analysis of Variance to determine any significant differences and Fisher's Least Significant Difference test to identify which were significant. A confidence level of 0.05 was used.

Table 8 APT agar (31)

Ingredient	Amount per litar
Agar	15 0 g
Pancreatic digest of casein	12 5 g
Glucoae	10 0 g
Yeast extract	7 50 g
NaCl	5 0 g
K_2HPO_4	5 0 g
Sodium citrate	5 0 g
Na_2CO_3	1 25 g
$MnCl_2*4H_2O$	0 14 g
$MgSO_4*7H_2O$	0 04 g
Thiamine*HCl	1 0 g

The pH ia adjuated to 6 7 ± 0 2 at 25°C Two percent fructoae or 2% glucoae and brom creoaole purple ia added to the medium-

Table 9 MRS (DeMan, Rogosa, Sharpe) agar (76)

Ingredient	Amount per litar
Glucosa	18.50 g
Peptona	10.0 g
Agar	10.0 g
Beef extract	8.0 g
Sodium acetata*$3H_2O$	5.0 g
Yeast extract	4.0 g
K_2HPO_4	2.0 g
Triammonium citrata	2.0 g
$MgSO_4*7H_2O$	0.20 g
$MgSO_4*4H_2O$	0.05 g
Sorbitan monooleata	1.0 ml

The pH is adjusted to 6.2 ± 0.2 at 25°C

Table 10 Yeast malt extract agar (YM) (31)

Ingredient	Amount per liter
Agar	20 0 g
Glucose	10 0 g
Agar	10 0 g
Peptone	5 0 g
Yeast extract	3 0 g
Malt extract	3 0 g

The pH is adjusted to 6 2 ± 0 2 at 25°C

MRS agar incubated aerobically was deemed to be the best all-purpose agar, therefore MRS agar (Oxoid lot # 31955337) was used for the remainder of the experiments

Biolog Method of Bacterial Identification

Cells were grown on MRS agar plates in pure culture to provide sufficient cell density to perform the analysis The manufacturer s recommended plating media was Biolog s Lactic Acid (BLA) agar but the media comparison studies determined that the lactic acid bacteria grew better on the MRS agar The plates were incubated for 24 – 48 hours at

25°C either aerobically or anaerobically depending on the demands of the organism

Cells were harvested by applying 2-3 ml of the BLA broth directly to the plate and dislodging the cells from the medium using a sterile loop A sterile dropper was used to collect the harvested cells and they were added to approximately 15 ml of sterile BLA broth The suspension was adjusted to an O.D at 590 nm of 0 60 to 0 70 using a Biolog Model 21101 spectrophotometer The manufacturer's recommended inoculum O D was 0 38 to 0 42, but preliminary experiments and industry experience indicated that a higher inoculum was necessary

One hundred and fifty microliters of BLA broth and cells were inoculated into each of the microwells in the Biolog plates The plates contained 96 wells, each with a dehydrated panel of nutrient medium, a carbon source, and tetrazolium violet Tetrazolium violet is a purple formazan, a redox dye, that turns purple when reduced, indicating use of the carbon source provided Each plate contained a positive and negative control well The plates were incubated at 25°C for 24 hours Four-hour readings are offered by the manufacturer, but were not possible due to

the slow growth of these bacteria The plates were placed into a Biolog automatic plate reader and the resulting pattern was analyzed using the Biolog program software. Identifications were made based on the carbon source utilization profile using the existing Biolog database for Gram positive organisms. Each carbohydrate utilization profile or pattern was stored in a custom library for future comparisons A dendrogram of the sample results was generated Figure 4 outlines the Biolog identification procedure

Fatty Acid Extraction for Identification

Samples were analyzed using the Microbial Identification System manufacturer's procedures Cells were grown on MRS plates for 24 to 48 hours at 28 ± 1°C under aerobic conditions A chart recorder was monitored to ensure that the exact growth temperatures were met The amount of growth was checked at 24 hours and, if a sufficient quantity was present, it was harvested If a sufficient quantity was not present, the plates were incubated for an additional 24 hours Cells were never grown for more than 48 hours These growth conditions were

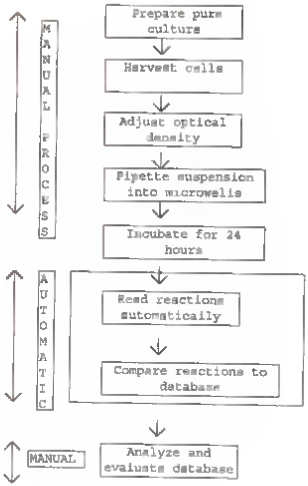


Figure 4 Process flow for Biolog analysis

strictly adhered to A sterile spatula was used to harvest the growth by scraping the surface of the media A minimum of 40 µg of pure culture growth was necessary to obtain results This translated to 1-2 plates of heavy growth or 3 or more plates of light growth The cells were placed in presterilized 13 x 100 screw cap test tubes The test tubes were hand washed, rinsed with regular tap water and then rinsed with distilled, deionized water prior to sterilizing

One milliliter of 1 2 M NaOH in 50% aqueous methanol was added to the cells to saponify the lipids This mixture was heated at 100°C for 30 minutes The mixture was cooled to room temperature and 2 ml of methylating reagent(325 ml of 6 N HCl and 275 ml of methanol) was added This was heated for 10 minutes at 80°C Again, the mixture was cooled to room temperature and 1 25 ml of the extraction reagent was added (200 ml of hexane and 200 ml of methyl tar butyl ether) The mixtures were mixed with a specimen tube rotator (Fisher catalog # 13 688-1D) at room temperature for 10 minutes The lower aqueous phase was removed and discarded and 3 ml of 0 3 M NaOH was added to clean the extract The mixtures were continuously mixed for 5 minutes at room temperature using a rotator Two-thirds

of the organic phase was transferred to a septum capped vial and placed in a refrigerator until analysis by gas chromatography. A calibration standard and a negative control were run with each batch. The calibration standard used was external and contained a mixture of straight-chain saturated fatty acids from 9 to 20 carbons in length and included five hydroxy acids. The hydroxy acids are used as quality control checks for the operator because they are sensitive to pressure and temperature fluctuations and also any contamination that may enter through the injection port (95).

The gas chromatograph used was a Hewlett Packard 5890 series with a flame ionization detector. The injected sample was volatilized and swept through the column by the carrier gas (hydrogen). The column was a fused silica capillary column (25 m x 0.2 mm) coated with 5% phenyl methyl silicone. The column was encased in a thermoregulated oven and the temperature was increased from 170 to 270°C at increments of 5°C per minute. The column was cleaned by heating to 310°C for 2 minutes after analysis. The flame ionization detector sends the electronic signals produced by the analytes to integrators

that amplify and process the signals The retention time of the various peaks are converted to chain length data and this data is used to make an identification Samples with a total peak area of <80,000 or <85% were repeated Identifications were made using the existing MIDI fatty acid profile database and the profile information was stored in a custom library for future comparisons A dendrogram of the sample results was generated Figure 5 outlines the fatty acid method.

Ribotyping Method of Identification

Cells were grown on MRS plates for 24 48 hours at 25°C either aerobically or anaerobically depending on the organism Two to three colonies were harvested using a stick or a loop and placed into 40 µl of lysis buffer (1 mM Tris HCl 20 mM EDTA - pH 8) The sample was then heated to 80°C for 10 minutes to neutralize the bacteria and intracellular enzymes that may interfere with the DNA digestion The heated samples were cooled, and lysostaphin (Sigma L7386) and lysozyme (or N-acetylmuramidase) were added to the cells The DNA was digested with *EcoRI*

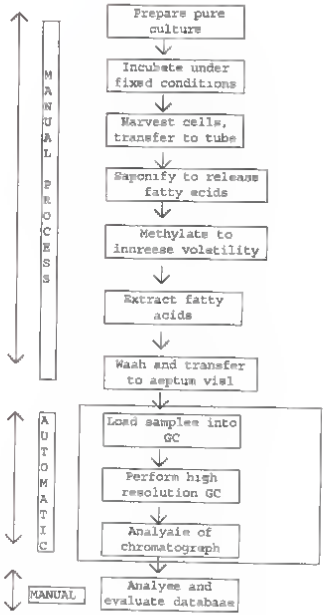


Figure 5 Process flow for fatty acid analysis

restriction enzyme and the fragments were separated using gel electrophoresis (0.8% agarose gel). Each gel contained thirteen lanes. Samples were run in lanes 2,3,5,6,8,9,11, and 13. Molecular weight standards were run in lanes 1,4,7,10, and 13. The weights of the standards were 0.99,2.2,3.2,6.35,9.6, and 48 Kbp. The gel was run for 65 minutes at 48.52V and 9-12 mA. The separated fragments were immobilized on a nylon membrane and denatured to separate the double stranded DNA to single stranded so that the probe can attach. The membrane was then exposed to a probe directed at a portion of the DNA coding for the ribosomal RNA. Specifically, the probe is directed at the 23S, 16S and 5S portions of *E. coli* DNA and is 6400 base pairs in size. This probe was sulfonated and a conjugate was added which attached to the sulfonated probe. Finally, a substrate was added which reacts with the conjugated probe to produce a chemiluminescent signal which was captured by the imaging capture system. This image was used to generate a picture of the signal. The fingerprint or riboprint was used to make an identification. The Dupont equipment did not have a database for the lactic acid bacteria, but the ATCC strains were repeated at least three times and, if the

patterns [illegible] a correlation of 0 93 or higher, the images were averaged and the print was stored in a database All images were stored for future comparison A dendrogram was generated using the computer assigned similarity coefficients Figure 6 outlines the ribioprinting process

Comparison of Methods for Identifying ATCC Strains

ATCC samples of the different strains were tested using all three methods Cells were grown on MRS plates and the same plate was used for all tests if possible Two to three colonies were picked and used for ribotyping From the growth left, the 40 µg necessary for the fatty acid determination was taken using a sterile spatula and placed in a sterile screwcap tube for extraction Finally, the remaining growth was harvested by placing approximately 5 ml of the Biolog broth directly on the plate A sterile suction pipette was used to suction off the broth now turbid with cells The cells were placed into a sterile large screwcap tube and diluted until the desired 0 6 to 0 8 O D at 590 nm was achieved After the ATCC strains were tested, a number of industrial isolates were analyzed These isolates were obtained from the cheese, meat, orange juice,

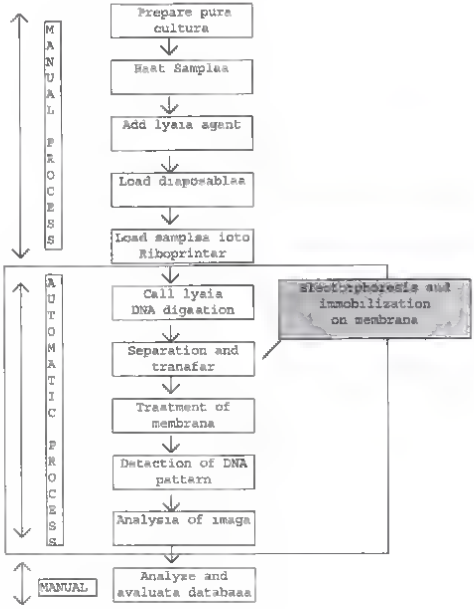


Figure 6 Process flow for Riboprinter

beverage, pet food, and beer industries

Results were plotted on a dendrogram to compare the methods of analysis in their similarity and differences Strains that appeared the same were retested using two other restriction enzymes, *HindIII* (Sigma R-1137 Lot 045H082) and *BamHI* (Sigma R-0260 Lot 94H0583)

Monitoring of Fermentations

The final stage of this research was to apply the "best" method or methods to the monitoring of a real food fermentation to determine if it would be a useful tool for this purpose The design of the three methods requires that all undergo an initial pre-growth stage prior to testing This does not meet the criteria of this research which is to develop a method to quickly identify a problem that may be occurring within a fermentation Direct analysis of the organisms within the fermentation may provide the speed necessary All three methods presented the potential for direct analysis, but the Riboprinter was chosen for reasons presented in the discussion section The Riboprinter was tested with mixed cultures from plates and broths to determine its ability to analyze these types of samples

Two to three strains were inoculated onto solid MRS agar and harvested by pulling a loop through the growth. For the broth tests, one to three strains were inoculated into MRS broth and incubated 24 to 48 hours at 24°C. The broth was decanted to concentrate the growth and an O.D. of 1.4 at 590 nm was used for ribotyping. Two fermentations were tested, sausage and sauerkraut. The sausage fermentation was performed using a starter culture, while the sauerkraut was a "natural" fermentation.

Sausage Fermentation Monitoring

Three pounds of ground beef with a 75:25 ratio of beef to fat was obtained. To determine the background ribotype patterns of the raw beef, a 1:5 dilution was made with peptone buffer and blended using a Tekmar Model STO400 Stomacher. Two milliliter portions of the peptone buffer were placed in high speed microfuge tubes and spun down for 10 minutes at 7000 x g using a microfuge (Fisher Model Micro V). The resulting pellets were combined and added directly to the sample buffer for the Dupont ribotyping machine. The experiment was repeated using a Stomacher bag with filter to remove large particulates such as the fat in the meat.

Manual Stomaching was also attempted Finally, the experiment was performed using a rinse of the meat The resulting pellets were combined and a loopful was added directly to the Riboprinter sample buffer. The pellet was simultaneously plated on SPC and MRS agar and incubated aerobically at 25°C for 24 hours

Three starter cultures were used to inoculate three separate sausages The cultures were grown in MRS broth at 25°C for 24 hours until turbid Table 11 lists the formulation used for the sausages

Table 11 Sausage formula

Ingredient	Amount(g)
Ground beef	908
Salt	62
Dextrose	45
Water	20
Pepper/spices	13
Sodium nitrite	1 5

The starter cultures used were *Pediococcus acidilactici*, *Pediococcus pentosaceus* and *Lactobacillus plantarum*. They were inoculated at a level of 10^4 organisms/gram of meat. The sausages were incubated at 25°C. Samples of the sausage were taken every 8 hours and analyzed by the method as described above.

Sauerkraut Fermentation Monitoring

Raw cabbage was purchased for this experiment. To determine the background patterns of the raw cabbage, a 1:5 dilution was made with peptone buffer and blended using a Tekmar Model STO400 Stomacher. Eight aliquots of 2 ml of the peptone buffer were placed in high speed ultracentrifuge tubes and spun down for 10 minutes at 7000 x *g* using a ultracentrifuge (Fisher Model Micro V). The resulting pellets were combined and added directly to the sample buffer for the Dupont riboprinting machine. The pellet was simultaneously plated on SPC and MRS agar and incubated aerobically at 28°C for 24 hours. No starter culture was added. The natural flora present on the cabbage was used for the fermentation. Salt was added to the cabbage at a level of 2.25%. The mixture was divided into four aliquots

and each was placed in a glass jar with a weighted plastic cover to prevent air from entering. The samples were incubated at 28°C and each week an aliquot was analyzed to monitor the fermentation

RESULTS AND DISCUSSION

Cultivation of Lactic Acid Bacteria

The lactic acid bacterial strains were taken from deep freeze (-80°C) and grown on MRS slants. The lactic acid bacteria, as a group, were difficult to culture, and when they did grow, the speed and amount of growth varied by the species. When taken directly from the deep freeze, the majority of the strains grew relatively quickly (24-72 hours) under aerobic conditions, but after the initial culturing a few strains did not grow again and many lost their viability with repeated transfers. At this point it would be an oversight to not mention how difficult it was to work with these organisms. Their growth rates differed from day to day. The original frozen cultures were subaliquoted for frequent access when the working cultures lost viability. It was also difficult to coordinate the timing of the experiments and the harvesting required for each. Rapid method was difficult because the bacteria failed to cooperate. Because these identification techniques

(particularly the fatty acid analysis) required harvesting growth at specific stages, culturing became a significant portion of the work for this thesis. In addition, since the amount of cellular material affected test result for the Biolog and fatty acid analysis, slow growing organisms were particularly challenging

Table 12 reports the visual appearances of the ATCC strains Each culture was stained and the morphology recorded All the cultures were Gram positive and catalase negative Fresh cultures stained well and were easily identified as Gram positive Some cultures greater than 72 hours old appeared Gram variable

A great deal of morphological diversity was noted within the lactic acid bacterial group Sometimes morphological differences can be attributed to the effect of the growth conditions however, the diversity noted with these organisms was consistent during repeated transfers To visually portray these morphological differences pictures of different members of this bacterial group were taken These are included in Figures 7 - 13 All cultures shown were grown for 48 hours at 28°C on MRS agar and were harvested in the logarithmic stage of growth

Table 12 Description of the ATCC strains

Organism Name	ATCC #	Description
Lactobacillus acidophilus	4356	small rods
Lactobacillus alimentarius	29643	short thick rods
Lactobacillus amylovorus	33620	thin rods in chains
Lactobacillus animalis	35048	thin rods
Lactobacillus bifermentans	35409	very short rods
Lactobacillus brevis	27305	var short rods in chains
Lactobacillus brevis	14869	short rods in chains
L casei ss casei	393	short thick rods
L casei ss rhamnosus	7469	thick rods
Lactobacillus collinoides	27612	short thin rods
Lactobacillus confusus	16881	short thick rods
L coryniformis ss coryniformis	25602	var short fat rods
L coryniformis ss torquens	25606	short fat rods
Lactobacillus curvatus	25601	var small rods
Lactobacillus delbrueckii	9649	small rods in chains
Lactobacillus delbrueckii	6089	long thin rods
Lactobacillus farciminis	29644	long thin rods
Lactobacillus fermentum	14931	short fat rods
Lactobacillus fructuous	13162	short thin rods
Lactobacillus halotolerans	35410	short small rods
Lactobacillus hilgardii	8290	short small rods
Lactobacillus heterofermentans	49373	long thin rods in chains
Lactobacillus minor	35412	small rods

Table 12 continued

Lactobacillus murinus	38928	long thin rods
Lactobacillus parabuchneri	49374	short rods in chains
Lactobacillus paracasei	28898	short thick rods
Lactobacillus paracasei	29392	short small rods
Lactobacillus pentosus	8041	short thick rods
Lactobacillus plantarum	14917	medium thin rods
Lactobacillus plantarum	8014	short fat rods in chains
Lactobacillus reuteri	23272	medium thin rods
Lactobacillus sake	15821	long thin rods in chains
L. mallvarius ex salicinius	11742	short rods in chains
Lactobacillus sumbicus	49379	short fat rods
Pediococcus acidilactici	33314	large cocci in tetrads
Pediococcus acidilactici	8081	cocci in tetrads
Pediococcus acidilactici	8042	med cocci in clusters
Pediococcus damnosus	29389	irr fat cocci in pairs
Pediococcus dextrinicus	33897	large cocci in clusters
Pediococcus pentosaceus	33316	small round cocci
Leuconostoc citreum	49378	irr cocci in pairs
Leuconostoc lactis	19256	irr cocci in tetrads
Leuconostoc mesenteroides	19255	small cocci in pairs
Leuconostoc mesenteroides	19254	small cocci in chains
Leuconostoc paramesenteroides	3113	irr cocci in pairs
Leuconostoc pseudomesenteroides	49371	small cocci
Lactococcus garvieae	43921	round cocci in clusters

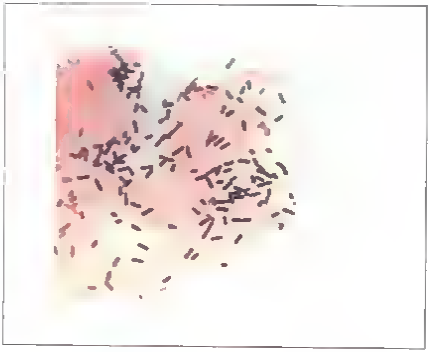


Figure 7 Medium Gram positive rods *L. brevis* (CL104) (x1000)

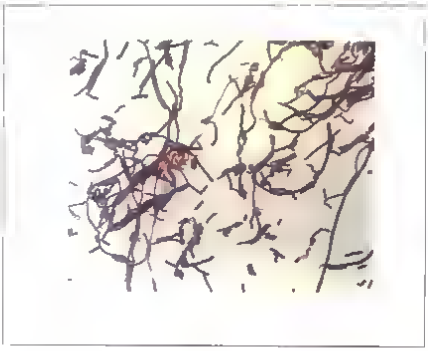


Figure 8 Long thin Gram positive rods - *L casei* (CL1
(x1000)

Figure 3 Thick Gram positive rode - *L. plantarum* (ATCC 8014) (x1000)

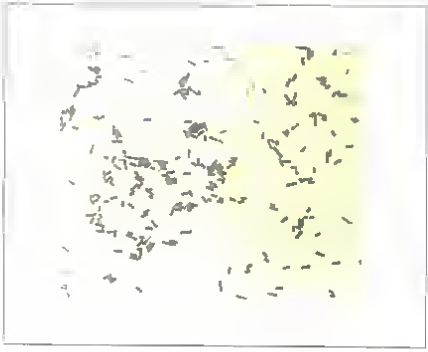


Figure 10. Small Gram positive rods *L. collinoides* (x1000)

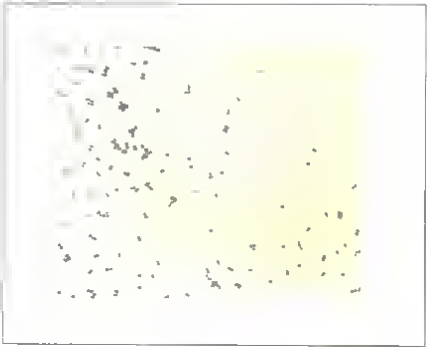


Figure 11 Small Gram positive cocci - *P. acidilactici* (UF16) (x1000)

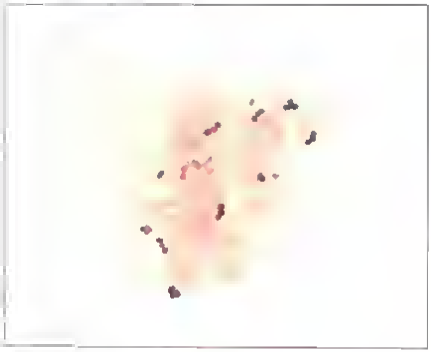


Figure [illegible] Irregular Gram positive cocci *L. citreum* (ATCC [illegible] x1000)

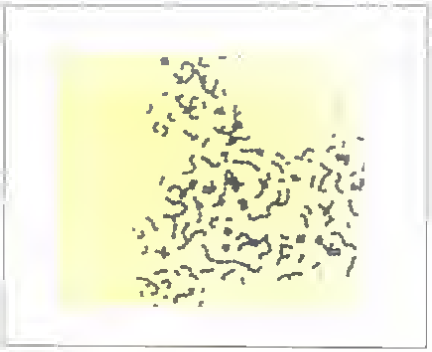


Figure 13 Small Gram positive cocci in chains - *L mesenteroides* (ATCC 19254) (x1000)

Plating Media Comparison

The results of the initial screening of the five plating media are shown in Table 13 Results are reported as the optical density at 590 nm of the growth harvested from the different plating media These results are also shown graphically in Figure 14 (the total of the optical densities for all strains was used for graphical purposes)

A One Way Analysis of Variance was used to determine that there was a significant difference between the plating media using a confidence level of 0.05 Fisher's Least Significant Difference test indicated that APT with sucrose, APT with fructose, and YM agar were significantly different from deMann, Rogosa and Sharpe (MRS) and Biolog Lactic Acid (BLA) agars MRS provided the greatest overall cellular quantity The Biolog Lactic Acid medium also performed well The Yeast and Mold (YM) agar was least successful The experiment was repeated two more times on two different days to verify the results However, for the second set of experiments ten lactic acid bacterial strains were selected and only MRS BLA and APT with 2% fructose (the three media with the greatest total O D at 590 nm) were used

Table 13 Plating media comparison[a] Initial screening

ATCC Strain	Plating Media (O D at 590 nm[a]) MRS	TMA	ELA	APT+f[b]	APT+s[b]
L acidophilus 4356	0 0[c]	0 0	0 0	0 0	0 0
L alimentarius 29641	1 7	0 2	1 7	1 6	1 4
L brevis 14869	1 9	1 6	1 8	1 8	1 7
L brevis 27305	1 7	0 3	1 4	1 5	1 1
L casei 393	1 6	0 3	1 5	0 4	1 1
L coryniformis 25602	0 0	0 0	0 0	0 0	0 0
L coryniformis 25600	0 4	0 1	0 3	1 4	1 2
L curvetus 25601	2 0	0 2	2 0	1 4	1 2
L farciminus 29644	2 0	0 02	-[d]	0 6	0 3
L fermentum 14931	0 02	0 04	0 03	0 06	0 0
L fructosus 13162	0 0	0 0	0 0	0 0	0 0
L halotolerans 35410	1 4	0 5	1 4	1 5	1 2
L malefermentans 49373	0 0	0 0	0 9	0 7	0 6
L murinus 35020	1 2	0 0	1 2	0 6	0 9
L parabuchneri 49374	0 9	0 05	0 6	0 6	0 2
L paracasei 25302	>2 0	1 7	1 9	1 8	1 7
L paracasei 25599	0 5	0 3	1 0	0 5	0 2
L pentosus 8041	0 8	0 7	0 8	0 6	0 6
L plantarum 14917	>2 0	1 7	1 0	1 6	1 7
L sake 15521	>2 0	1 4	1 5	1 2	1 0
Leu citreum 49370	>2 0	>2 0	>2 0	>2 0	>2 0
Leu lactis 19256	2 0	1 0	1 9	1 0	1 0
Leu pseudomes 49371	1 9	1 0	1 9	1 0	1 0
P acidilactici 33314	2 1	1 0	1 5	1 15	1 15
P damnosus 29358	0 0	0 0	0 0	0 0	0 0
P dextrinicus 13887	0 0	0 0	0 0	0 0	0 0
P urinaequi 29723	0 05	0 05	0 17	1 35	0 0
Lactococcus garvieae 43921	0 7	0 5	0 7	0 4	0 5
Lactococcus lactis 29071	0 0	0 0	0 0	0 0	0 0
Average O D reading	1 12	0 50	1 0	0 92	0 81
Total O D reading	32 47	16 86	28 00	26 56	23 15

[a] Optical density performed on a suspension of cells washed from each plating medium

[b] APT agar with 2% fructose (APT+f) or 2% sucrose (APT+s)

[c] Indicates no growth

[d] Sample lost

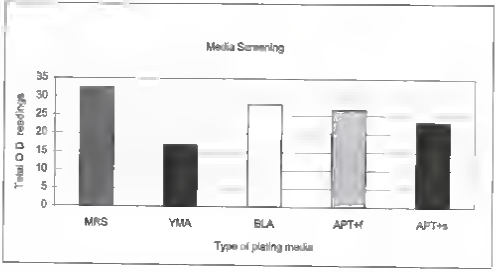


Figure 14 Comparison of plating media- Initial screening of ATCC strains by O D $_{595\ nm}$ measurements of cell density

The results are summarized in Table 14 and are graphed in Figure 15. The results are reported, again, as optical density at 590 nm and the total growth from each of the ten strains provided values for each different plating medium.

Table 14: Comparison of plating media: Final comparison of ATCC strains

ATCC Strain	Plating Media (O.D. results at 590nm)[a] MRS	MRS	SLA	SLA	APT+f[b]	APT+f[b]
L. brevis 27305	1.1	0.8	0.8	1.1	0.4	0.5
L. alimentarius 29041	1.1	0.7	0.9	0.9	0.8	0.7
L. casei 191	0.6	0.3	0.4	0.1	0.5	0.6
L. coryniformis 25600	0.4	0.6	0.1	0.4	0.2	0.8
L. farciminus 29644	1.1	0.7	0.8	0.9	0.1	0.4
L. murinus 35020	1.2	1.2	0.8	0.9	0.7	0.3
L. parabuchneri 49174	0.5	0.5	0.8	0.8	0.2	0.1
Leu. pseudomes 49171	0.7	0.5	0.8	1.0	0.8	0.8
P. acidilactici 11114	1.1	1.0	1.5	1.4	1.1	0.9
Average O.D. reading	0.78		0.77		0.51	
Total O.D. reading	14.1		13.8		9.1	

[a] Optical density performed on a suspension of cells washed from each plating medium

[b] APT agar with 2% fructome (APT+f)

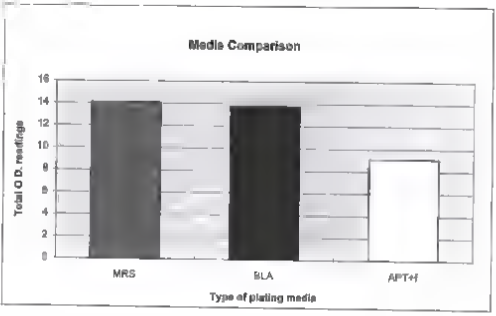


Figure 15 Final plating media comparison by O D $_{590\ nm}$ measurements of cell density

Once again, MRS agar produced the greatest total cellular quantity however, Fisher's Least Significant test showed that there was no significant difference between the MRS and the BLA agars based on the results to these experiments. The APT agar with sucrose was significantly lower. The MRS agar was selected for use in the remaining experiments since it is applicable to all three identification techniques and it is a commonly used medium for lactic acid bacteria throughout the industry. It is marketed as a commercial formula and can be obtained from Oxoid with relative ease.

Biolog

The performance of the different identification techniques was evaluated using ATCC versions of strains that are listed in the database of the individual test. Evaluations of all strains on each test were not possible due to the variations of the organisms and their applicability to each test. An attempt was made to evaluate as many as possible for comparison purposes.

Table 15 lists the strains that were used to evaluate the performance of the Biolog test. These are ATCC strains

Table 15 ATCC strains tested by Biolog

Lactobacillus alimentarius	*Lactobacillus animalis*
Lactobacillus bifermentans[a]	**Lactobacillus brevis** *(2)*[b]
L. casei ss casei	**L. casei ss rhamnosus**
Lactobacillus curvatus	*L. coryniformis ss coryniformis*
Lactobacillus delbrueckii	*Lactobacillus coryniformis*
Lactobacillus confusus	*Lactobacillus fermentum*
Lactobacillus farciminus	*Lactobacillus halotolerans*
Lactobacillus murinus	*Lactobacillus malefermentans*
Lactobacillus minor	**Lactobacillus paracasei** *(2)*[b]
Lactobacillus pentosus	*Lactobacillus parabuchneri*
Lactobacillus plantarum *(2)*[b]	*Lactobacillus reuteri*
L. salivarius ss salicinius	**Pediacoccus pentosaceus**
Pediococcus acidilactici *(2)*[b]	*Pediococcus dextrinicus*
Leuconostoc citreum	**Leuconostoc mesenteroides**
L. paramesenteroides	*L. pseudomesenteroides*

[a] Strains in bold were tested by all identification methods

[b] Two different ATCC strains of these organisms were used

and are only those species that the Biolog manufacturers claim can be identified by their test Table 16 provides the percent of correct responses for those organisms tested

These results show that 25 species (47 2%) were incorrectly identified meaning that the ATCC derived designation was not the first choice when using the Biolog For 10 of the incorrectly identified strains, the genus identification was correct For 5 of these incorrect strains correct identification was given as one of the top three choices For the other 20 strains, the correct designator was not listed among any of the choices The Biolog did not identify 12 (22 6%) of the strains It was interesting to note that 2 samples gave the correct identification after 72 hours of incubation

Table 16 Correct responses for Biolog identifications of ATCC strains

# Strains	Genus id	Species id	Subspecies id
33[a]	71 7%	52 8%	100%[b]

[a] 53 total samples
[b] The three strains designated to subspecies were correctly designated as such

The Biolog procedure was easily executable once growth on solid plating media was obtained. Results are calculated based on the reactions in the multiwell plate. These reactions are converted to a number based on the well reaction. This number is then used to best fit the strain being tested into a category or group that reacts similarly. One problem is that the variation or prevalence of a strain based on environmental conditions of the isolate may be missed (9). One author (10) recommends the application of Bayesian analysis to make a final determination. This author agrees that the usefulness of this tool is great when applied with background information and knowledge of the sample. For example, the Biolog program has a media selection key. If the type of medium is changed on the menu, the identification may be different even though the reactions are identical. This illustrates the need for an educated user and concurrent testing to determine the correct classification into which the organism belongs. Other considerations are that the Biolog requires a large cell quantity of a pure culture and incubation of the plates for lactic acid bacteria is 24 hours minimum. The condition of the cells and the type of growth media may affect the

reactions in the plate and, finally, slow growing organisms were difficult to analyze in both the harvesting stage and in the microwell plate Slow growers will give false negative reactions In numerous instances 48 to 72 hour results provided better information For the purposes of these experiments, the amount of cellular material inoculated into the plates was increased from the manufacturer's suggested O.D. (0.40) to 0.6 to 0.7 in an attempt to overcome the issue of slow growing organisms

Fatty Acid Analysis

Table 17 lists the strains that were used to evaluate the Microbial Identification System that uses fatty acid analysis as a means of identifying the organisms Again, these were ATCC strains and are only the species that the MIDI manufacturers claim can be identified by their test Table 18 provides the percent of correct responses for those organisms tested

The results show that 22 strains (73.3%) were incorrectly identified meaning that the ATCC derived designation was not the first choice when using the MIDI system Of the 22 strains that were incorrect, 21 (70%) of

Table 17 ATCC strains tested by MIDI

Lactobacillus bifermentans[a]	**Lactobacillus brevis** (2)[b]
Lactobacillus casei ss casei	**L. casei ss rhamnosus**
Lactobacillus confusus	**Lactobacilles fermimisus**
Lactobacilles paracasei (2)[b]	**Lactobacillus plantarum**
Pediococcus pentosaceus	**Pediococcus acidilactici** (3)[b]
Leuconostoc mesenteroides	L paramesenteroides
L **pseudomesenteroides**	

[a] Strains in bold were tested by all identification methods
[b] The number in parentheses indicates if more than one ATCC strain was tested for that species

Table 18 Correct identifications by MIDI using ATCC strains

# Samples	Genus id	Species id	Subspecies id
18[a]	71 4%	25 0%	100%[b]

[a] 28 total samples
[b] One strain was designated to subspecies and was correctly identified as such.

the strains were correctly identified to the genus only. The Microbial Identification System was unable to identify 3 (10%) strains and produced a "no match" result for these. Interestingly, in the case of the *Leuconostoc* species, *L. pseudomesenteroides* and *L. paramesenteroides* were identified in reverse. Likewise, *Lactobacillus paracasei* was identified as *Lactobacillus casei*.

The extraction procedure for the fatty acid analysis has been reduced to a single tube extraction which is not difficult, but it is lengthier than the sample preparation for the other methods (104). Because it involves, first, a reaction with methanol in the presence of an acid catalyst to form the methyl ester and, then, an extraction with timely steps, it is impractical to devote that time to a small number of samples and laborious to do too many at once (118). Twelve samples at a time was manageable in this author's perspective. Some work to automate the sample preparation has been published, but is not widely applied (49). The cells must be completely saponified in a boiling water bath and the time and temperature during methylation are critical. For some strains of lactic acid bacteria,

obtaining the quantity of cells necessary for harvesting was difficult

If the analysis of cellular fatty acids is the main technique for identification, great care must be placed to control the variables such as culture media and temperature as well as time of incubation This is essential especially when cultures are to be differentiated at the species or subspecies level For qualitative results, tight control is not as necessary because many of the major fatty acid components tend not to vary, just their relative amounts There exists a great deal of literature discussing the analysis of bacteria using fatty acid profiling Advances in chromatographic techniques have allowed this technique to become more accurate For example, the use of a fused silica capillary column provides the opportunity to distinguish between iso and anteiso isomers of the same carbon length and also allows the resolution of hydroxy derivatives (111) However, the literature varies with respect to the performance of this method

One report discussing the use of fatty acid analysis for the identification of Staphylococci noted that species of these organisms could be distinguished only by the use of

cluster analysis In the analysis of non fermenting Gram negative bacteria, qualitative analysis of the fatty acids was used as an additional method for species differentiation and in some cases as a method for routine identification (111) Similarly, qualitative data was used to group clinical strains of *Pseudomonas capacia* for epidemiological purposes (69)

Birnbaum et al (12) used the MIDI to compare *Staphylococcus* isolates from clinical sources and found that it could be useful as a screening tool for epidemiologic purposes although, in one instance, it failed to correctly cluster a group of isolates that were identical using whole plasmid analysis and several analyses of chromosomal DNA This suggests that the fatty acid method may not be as definitive as genetic methods The use of cellular fatty acid analysis has proven useful in distinguishing streptococci which possess $C_{16:1\omega7c}$ while members of *Enterococcus*, *Pediococcus*, *Aerococcus*, and *Lactococcus* do not In general, the use of fatty acid analysis as a highly discriminatory tool for typing was not recommended by these authors (115) In a study that compared the Microbial Identification System to conventional methods to identify

Gram negative anaerobic bacilli, 93.3% of the samples were correctly identified to the genus level but only 51.2% to the species level (104). Wauthoz et al. (114) recommended incubating the cultures for 48 hours to allow them to reach the stationary phase where the fatty acid profile is rather stable. They also found that reproducibility of the extraction procedure was best for organisms with hydroxy and cyclic fatty acids. *Lactobacillus plantarum* has a high cyclic fatty acid proportion which may account for the fact that in this research, it was the most reproducible test organism. It's relatively quick growth rate may be another factor. These researchers (114) also demonstrated that there may be differences for one particular fatty acid within strains of the same species. For *Lactobacillus plantarum* they calculated differences of 11.52% in palmitic acid within the same specie. This type of trend was also discovered within species of other genera such as *Staphylococcus* and *Bacillus*. Another factor that affected results was the physiological age of the culture rather than the incubation time alone. Furthermore, growth conditions played an important role in explaining differences in fatty acid profiles. Differences in the fatty acid profiles for

Staphylococcus epidermidis after 24 hours of growth were found to be caused by the presence of fermentable sugars in the media that caused biphasic growth conditions and natural kinetic differences within species. Since the lactic acid bacterial group ferment available sugars to produce lactic acid, they may not be an optimal group for the application of this technique. Growth temperature too, affected results. As a result, Wauthoz et al. (114) recommended that cultures be grown at or below optimum. Different strains of the species *Lactococcus lactis* could not be distinguished at 30°C, but if grown above the optimum (at 37°C), two distinct profiles were observed. Sensitivity was limited to the species level and, in addition, the profile "seldom appeared to be specific to a single species" (114: 1238). They reported best success with this method with *Bacillus* strains where typing is based on the presence or absence of particular fatty acids as opposed to relative proportions. When growth conditions with *Bacillus* were altered, the strain clustering did not change significantly. Gilarova et al. (40) reported that a lower than optimal cultivation temperature resulted in lower production of lactobacillic acid (cyc $C_{19:0}$) and a lower growth rate. These authors

tested a number of cultivation conditions on two species of lactic acid bacteria, *Lactobacillus saka* and *Lactobacillus pentosus*. Decreasing pH was found to increase the cyclopropyl content (cyc $C_{19:0}$) and increased salt concentration increased the ratio of saturated to unsaturated acids. Again, this supports the theory that the acids generated during fermentation may affect the identification.

Wauthoz et al. (114) reported that their lactic acid bacteria clustering data was in good agreement with other classification methods such as DNA base composition, DNA reassociation, and fermentation profiles using API strips. They suggested that the proper use of fatty acid identification for lactics be as a tool to confirm the results of other methods. They did conclude, however, that the test was useful for lactics because there are few alternatives for easy identification of lactics, unlike more clinically predominant species such as *Staphylococcus* and *Salmonella*. For these two genera, they found the test useless. Their general conclusion was that GC analysis of fatty acid composition is not currently specific to a single species. Taxonomic conclusions based on this analysis alone

may not be claimed The method of characterization by fatty acid profile should ba used as a complement to other taxonomic criteria in a scheme of polyphasic identification Gilarova et al (40) concluded that it was difficult to discern batween inherent properties of a cell and those properties that were caused by the effects of cultivation or previous cell history They determined standardization to be the main problem and this did not allow the comparison of results between laboratories

Ribotyping

The performance of the Riboprinter was based on the analysis of ATCC strains, but at the time of this research there was no developed database for the lactic acid bacterial group The database was generated by analyzing known ATCC strains and when two patterns matched to a correlation of 0 93 or better the pattern was named for the organism For the purpose of analysis, a match of 0 80 or higher was deemed acceptable for genus and species and 0 70 to 0 80 was deemed acceptable for genus only Table 19 lists the ATCC strains used for the evaluation and Table 20 shows the results

Table 19. ATCC strains tested on the Riboprinter

Lactobacillus alimentarius	*Lactobacillus animalis*
Lactobacillus acidophilus	*Lactobacillus amylovorus*
Lactobacillus bifermentans[a]	***Lactobacillus brevis*** *(2)*[b]
L. casei ss casei	***L. casei ss rhamnosus***
Lactobacillus curvatus	*L coryniformis ss coryniformis*
Lactobacillus collinoides	***Lactobacillus confusus***
Lactobacillus coryniformis ss torquens	*Lactobacillus coryniformis ss coryniformis*
Lactobacillus curvatus	*Lactobacillus fructosus*
Lactobacillus delbrueckii	*Lactobacillus fermentum*
Lactobacillus farciminus	*Lactobacillus halotolerans*
Lactobacillus hilgardii	*Lactobacillus malefermentans*
Lactobacillus murinus	***Lactobacillus paracasei*** *(2)*[b]
Lactobacillus pentosus	*Lactobacillus parabuchneri*
Lactobacillus plantarum *(2)*[b]	*Lactobacillus sake*
Lactobacillus suebicus	***Pediococcus pentosaceus***
Pediococcus acidilactici *(3)*[b]	*Pediococcus damnosus*
Leuconostoc citrum	***Leuconostoc mesenteroides*** *(3)*[b]
L. pseudomesenteroides	

[a] Strains in bold were tested by all identification methods

[b] The number in parentheses indicates if more than one ATCC strain was tested for that species

Table 20- Correct responses for Riboprinter identifications of ATCC strains

# Strains	Genus id	Species id	Subspecies id
40*	82 4%	74 5%	70 0%

* 102 total samples

These results show that 26 (25.5%) were incorrectly identified meaning that they did not match the ATCC derived pattern group when compared to the database- For eight of the unidentified strains, the genus identification was correct For another eight of the unidentified incomplete digestion of the DNA was the reason for the lack of identification For four, overloading of the gel was the reason and for two, cellular endonuclease activity within the cell caused extra bands This may often be seen in older cultures It was interesting to note that all of the strains that did not match were visually similar to the pattern group- Finally, 34 (33 3%) of the samples matched at 0 93 or higher which is considered the same clonal group

Factors that affected the results of the riboprinting included overdosing of the gel Overdosing resulted in the presence of many bands that were not seen with optimal DNA levels These extra bands resulted in the creation of a new

pattern group rather than being identified correctly This highlighted a weakness in the computer analysis of the patterns Specific bands are not weighted according to genus and species, therefore, environmental isolates that have slight genetic differences, but are clearly the same genus and, possibly, the same species are not identified as such Figure 16 provides an example of overloading of the gel for *Lactobacillus plantarum*

Another factor that led to poor results was the use of cultures in the late lag phase that had already begun to autodigest, forming many smaller bands not present in healthy cellular DNA Figure 17 shows extreme examples of autodigestion in an older cultures of *L. murinus* and *P pantosaceous* Finally, incomplete digestion continued to be the major source of poor results Figure 18 shows an example of incomplete digestion of *Lactobacillus amylovorus* when compared to a complete digestion of the same strain Even though the patterns did not meet the criteria for a match, it is easy to see that the band patterns are very similar

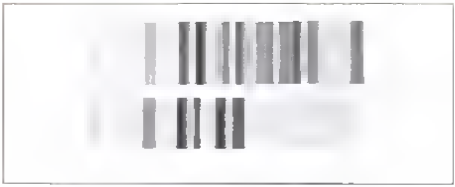


Figure 16 Overdosing of gel (top) versus normal loading for *Lactobacillus plantarum*

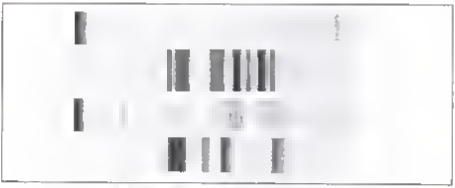


Figure 17 Stationary phase culture patterns (from top to bottom) of *Lactobacillus murinus* versus log phase (4th track) and *Pediococcus pentosaceus* versus log phase

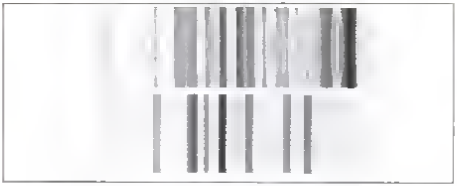


Figure 18 Incomplete digestion (top) of *L amylovorus* versus complete digestion

Figure 19 gives an example of matching patterns to illustrate the repeatability of the test Figure 20 shows the computer generated image of the gel This allows the researcher to measure the bands against the molecular weight markers or compare them visually if necessary

During initial experiments lysis of the lactic acid bacteria was not successful It was found that the addition of N-acetylmuramidase facilitated the lysis (52) Since this research spermine has been added to the protocol to enhance the performance of these samples by aiding in the precipitation of the DNA (19)

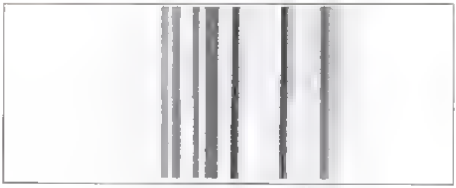


Figure 19 Matching patterns for *L. alimentarius*

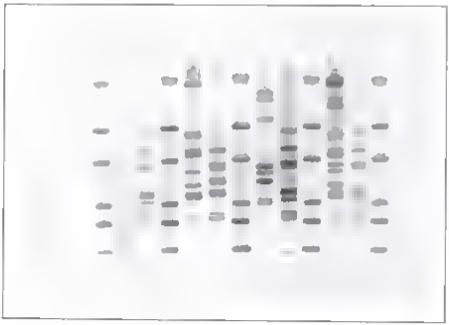


Figure 20 Computer generated image of gel with samples UF27, ML6, UF72, 620 UF33, 7378, UF27, UF34 in sample lanes 1-8 Controls are run in the first well and after every two sample lanes

Comparison of Biolog, Fatty Acid Analysis and Ribotyping

Dendrograms of ATCC Strains

Figure 21 shows the dendrogram of the *Leuconostoc* strains as determined by the Biolog. The *L. citreum* and *L pseudomesenteroides* strains clustered most tightly in this analysis, but in the Biolog database *L. citreum* was not in the same group as *L. mesenteroides*. Figures 22 and 23 show the dendrograms of *Leuconostoc* strains for the fatty acid analysis and ribotyping.

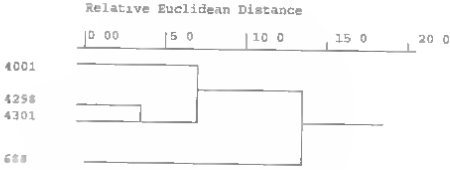


Figure 21 Biolog results for *Leuconostoc* spp.

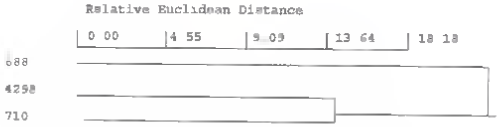


Figure 22 MIDI fatty acid results for *Leuconostoc* spp.

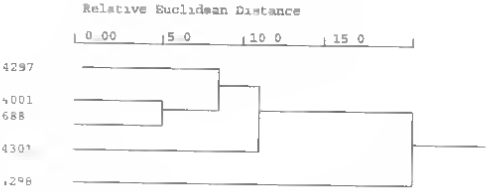


Figure 23 Ribotyping results for *Leuconostoc* spp.

Leuconostoc mesenteroides (4001) and *Leuconostoc paramesenteroides* (688) clustered very tightly in the ribotyping dendrogram, but did not in the Biolog *Leuconostoc pseudomesenteroides* (4298) was not clustered closely by ribotyping, but showed closer association to the other strains using Biolog and fatty acid analysis. This illustrates the differences between organisms when the analysis is biochemical versus genetic. See the appendix for the actual patterns for comparison. Figures 24, 25, and 26 show the dendrograms for the analysis of *Pediococcus spp* using the Biolog, Microbial Identification System for fatty acid and the Riboprinter for ribotyping, respectively.

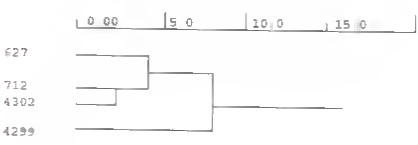


Figure 24. Biolog results for *Pediococcus spp*.

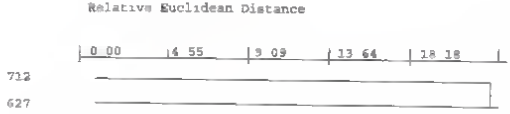


Figure 25. MIDI fatty acid results for *Pediococcus* spp

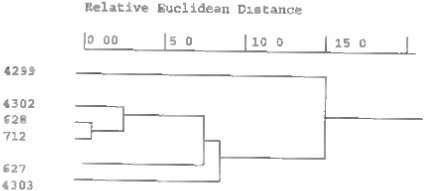


Figure 26 Riboprinter ribotyping results for *Pediococcus* spp

It is interesting to note, in all three analyses, *Pediococcus acidilactici* (strain 627) did not cluster closely to any strains even though strains 4302 and 628 are also *P. acidilactici* strains. This suggests that the strains are not as closely related as their names suggest.

The Biolog method for identifying members of the genus *Leuconostoc* makes the identification with only a small percentage of the total wells reacting. The ribotyping results for many of the *Leuconostoc* species has few bands relative to most members of the genus *Lactobacillus* and some of the *Pediococcus* species. This information is important to note when applying these methods to unknown strain characterization.

Figures 27, 28 and 29 provide the results for the analyses of the *Lactobacillus* strains. The results varied from test method to test method for these organisms. Some general trends were apparent though. *L. casei ss rhamnosus* (620) and *L. pentosus* (7372) clustered closely with fatty acid analysis and ribotyping, but not with Biolog. Both *L. plantarums* (625 and 7373) clustered closely on all three methods. *L. confusus* (7355) did not cluster closely by any method. Strains highlighted in bold are common to all three procedures.

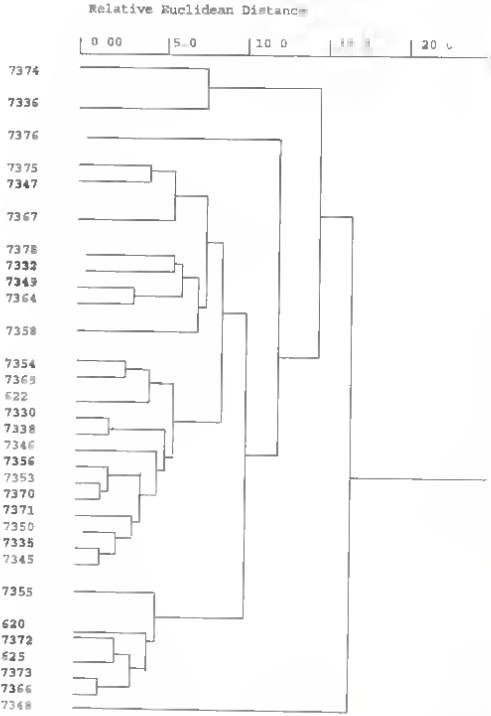


Figure 27 Biolog results for *Lactobacillus* spp

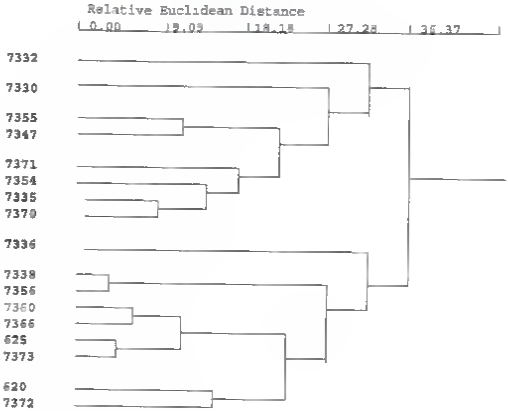


Figure 28 Fatty acid results for *Lactobacillus* spp.

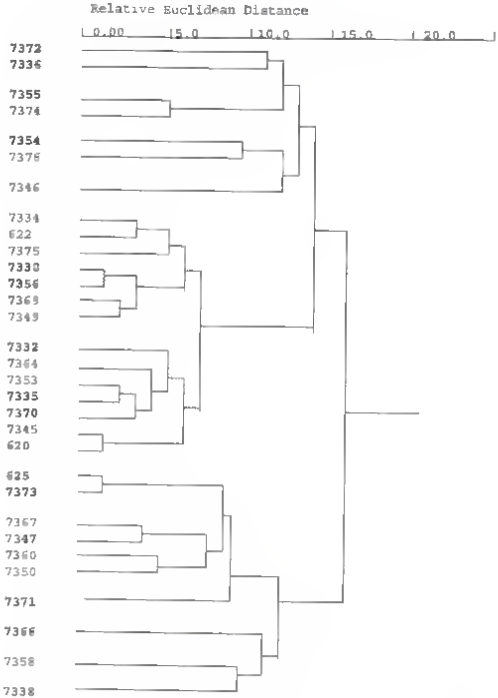


Figure 29 Ribotyping results for *Lactobacillus* spp

Analysis of Industrial Unknowns

Table 21 shows the results from the traditional testing and, in some cases, preliminary identifications of the industrial isolates. Table 22 shows the results of the industrial strain isolates using the three methods of identification.

In Table 22, if an isolate is listed in parentheses, the organism tested by ribotyping matched the organism in parentheses identically, but failed to match any in the database. "Not tested" indicates strains that were not run on that test for a variety of reasons including equipment failure, duplication by other samples, loss of strain, and inadequate growth. "No results" indicates samples that were attempted, but repeatable results were not obtained. Typically these samples failed to identify to the genus level consistently or gave insufficient results. For Biolog this was usually no reaction in the wells or, for ribotyping, no band pattern could be seen.

Not all isolates were tested by the Microbial Identification System. Since the performance of the MIDI had not been optimal with the ATCC strains, a decision was made to not run all the industrial strains by this method.

Table 21 Preliminary identification of strains

Source	Number	Identification
Dairy	KR5	*Enterococcus faecalis*
Dairy	KR1	*Enterococcus faecium*
Dairy	KR309	*Lactococcus cremoris*
Dairy	KR308	*Lactococcus lactis*
Dairy	KR141	*L mesent ss mesent*
Dairy	KR142	*L mesent ss dextran*
Dairy	KR137	*L mesent ss cremoris*
Dairy	KR143	*L mesent ss dextran*
Dairy	KR27	*Enterococcus faecalis*
Dairy	KR317	*Lactococcus lactis*
Dairy	KR18	*P acidilactici*
Dairy	KR22	*P pentosaceus*
Dairy	KR19	*P acidilactici*
Dairy	KR23	*P pentosaceus*
Dairy	KR488	*L casei ss casei*
Dairy	KR495	*L casei ss pseudoplant*
Dairy	KR316	*Lactococcus lactis*
Dairy	KR6	*Enterococcus faecalis*
Dairy	KR489	*L casei ss pseudoplant*
Dairy	KR494	*L casei ss rhamnosus*
Beer	ML6	*Lactobacillus spp*
Beer	ML7	*Lactobacillus spp*
Beer	ML8	*Lactobacillus spp*
Beer	ML5	*Lactobacillus lactis*
Beer	ML2	*L delbrueckii*
Beer	ML4	*L parscasei*
Beer	ML1	*L delbrueckii*
Orange juice	UF30	*L paramesenteroides*
Orange juice	UF71	*L mesenteroides*
Orange juice	UF34	*L farciminus*
Orange juice	UF18	*Lactobacillus fermentan*
Orange juice	UF16	*L mesenteroides*
Orange juice	UF10	*Leuconostoc dextranicum*
Orange juice	UF33	*Leuconostoc rhamnosus*
Orange juice	UF27	*Lactobacillus plantarum*
Orange juice	UF72	*L maltaromicus*
Orange juice	UF	*L mesenteroides*
Orange juice	UF26	*L mesenteroides*
Orange juice	UF17	*L mesenteroides*
Orange juice	UF16	*L mesenteroides*
Bakery	BAK5	*L mesenteroides*
Bakery	BAK14	*Lactobacillus plantarum*

Table 21 -continued

Orange juice	UF24	*L. paramesenteroides*
Orange juice	UF29	*L. paramesenteroides*
Orange juice	UF20	*L. paramesenteroides*
Orange juice	UF23	*L. paramesenteroides*
Orange juice	UF66	*Leuconostoc lactis*
Orange juice	UF33	*L. mesenteroides*
Orange juice	UF36	*L. mesenteroides*
Orange juice	UF00	*L. mesenteroides*
Orange juice	UF42	*L. paramesenteroides*
Orange juice	UF15	*L. mesenteroides*
Orange juice	UF52	*L. mesenteroides*
Orange juice	UF63	*L. mesenteroides*
Orange juice	UF30	*L. mesenteroides*
Orange juice	UF65	*L. mesenteroides*
Unknown	CCL118	*Lactobacillus spp*
Unknown	CCL124	*Lactobacillus spp*
Unknown	CCL102	*L. cellobiosus*
Unknown	CCL103	*L. acidophilus*
Unknown	CCL105	*Lactobacillus brevis*
Unknown	CCL106	*Leuconostoc lactis*
Beverage	CCL109	*Lactobacillus spp*
Ice cream	CCL123	*Lactococcus*
Unknown	CCL127	*Lactobacillus plantarum*
Unknown	CCL126	*Lactobacillus brevis*
Orange juice	CCL122	*Lactococcus spp*
Beverage	CCL112	*Lactobacillus spp.*
Beverage	CCL113	*Lactobacillus spp.*
Beverage	CCL110	*Lactobacillus spp*
Unknown	CCL100	*L. fructovorans*
Unknown	CCL101	*Lactobacillus casei*
Unknown	CCL104	*Lactobacillus buchneri*
Pet Food	CCL116	*Lactobacillus spp.*
Unknown	CCL121	*Lactobacillus casei*
Meat	OMLA2	Heteroferm lactic
Meat	OMLA7	Heteroferm lactic
Meat	OMLA9	Heteroferm lactic
Meat	OMLS7	Heteroferm lactic
Meat	OM	*Lactobacillus spp.*
Bakery	BAK10	*Lactobacillus casei*
Bakery	BAK19	*Lactobacillus plantarum*

Table 22 Results to identifications of industrial strains

#	Biolog	MIDI	Riboprintar
KR5	No results	*L pseudomes*	*Enterococcus faecalis (KR 8)*
KR1	Tetragenococcus halophilus	*L plantarum*	*L paracasei*
KR389	Streptococcus mitis	*L plantarum*	KR316 No name
KR388	Streptococcus suis	*L plantarum*	*Lactococcus lactis (KR317)*
KR317	No results	*L plantarum*	*L lactis(KR388)*
KR141	*L citrium*	*L pseudomes*	*Leucnostoc spp*
KR142	*L bifarmentans*	*L parames*	*Leucnostoc spp*
KR143	No results	Not tested	*Leucnostoc spp*
KR137	No results	*L mesenter*	*L mesentar*
KR27	No results	*L plantarum*	*Enterococcus faecalis*
KR18	No results	*P pentosaceus*	*P acidilactici*
KR19	*L bifarmentans*	*P pentosaceus*	*P acidilactici*
KR23	No results	*P pentosaceus*	*P pentosaceus*
KR488	*P dextrinicus*	No match	No match
KR495	No results	*L bifarmentans*	*L paracasei (ML4)*
KR316	Streptococcus mitis	*L mesent ss mes*	KR389 No name
KR8	No results	*L plantarum*	*Enterococcus faecalis (KR5)*
KR485	No results	*L bifarmentans*	*L paracasei (KR495 ML4 CL103)*
KR484	No results	*L casei*	*L casei*
ML6	*L buchneri*	*L buchneri*	*L brevis*
ML7	No results	*L brevis*	*L brevis*
ML8	No results	No results	*L brevis*
ML5	No results	Not tested	*L casei*
ML4	Not tested	Not tested	*L paracasei (KR495 KR485)*
ML1	Not tested	Not tested	*P damnosus*
UP30	*L gasseri*	Not tested	*P acidilactici*
UP71	Not tested	Not tested	*P acidilactici*
UP34	No results	*L confusus*	*L plantarum*
UP18	Not tested	Not tested	*L plantarum*
UP16	Not tested	Not tested	*P acidilactici*
UP10	Not tested	Not tested	*P acidilactici*
UP33	*L plantarum*	*L brevis*	*L plantarum*
UP27	*L pentosus*	Not tested	*L plantarum*
UP72	Not tested	Not tested	*P acidilactici*
UP26	*L mali*	Not tested	*P acidilactici*
UP17	*L hainlolerans*	Not tested	Not tested
UP16	*L mali*	Not tested	*P acidilactici*
RAK5	Not tested	*L mesent ss mes*	*Leucnostoc spp*
RAK14	Not tested	*L plantarum*	*L plantarum*
UP24	*L bifarmentans*	Not tested	*P acidilactici*
UP28	*L mali*	Not tested	*P acidilactici*
UP20	*L bifarmentans*	Not tested	*P acidilactici*

Table 22--continued

UF23	*L. bifermentans*[3]	Not tested	*P. acidilactici*[3]
UF65	Not tested	Not tested	*P. acidilactici*[3]
UF69	Not tested	Not tested	*P. acidilactici*[3]
UF66	*L. mali*	Not tested	*P. acidilactici*[3]
UF71	Not tested	Not tested	*P. acidilactici*[3]
UF90	Not tested	Not tested	*P. acidilactici*[3]
UF92	Not tested	Not tested	*P. acidilactici*[3]
UF93	*L. bifermentans*	Not tested	*P. acidilactici*[3]
UF96	*L. mali*	Not tested	*P. acidilactici*[3]
UF94	*L. pseudomes*	Not tested	No results
UF15	Not tested	Not tested	*P. acidilactici*[3]
UF42	Not tested	Not tested	*P. acidilactici*[3]
CL119	No results	Not tested	*L. collinoides*[3] (119, 122, 123, 126)
CL124	No results	Not tested	No results
CL102	*L. paracasei*[3]	Not tested	No match
CL103	*L. mesent cremoris*	Not tested	*L. paracasei*
CL135	No results	Not tested	*L. brevis* (CCL108, 164)
CL136	*L. casei casei*	Not tested	*L. casei casei*[3]
CL109	*L. mali*	Not tested	No results
CL121	*Lactococcus lactis lactis*	Not tested	*Enterococcus faecium*[3]
CL127	*L. plantarum*	Not tested	*L. plantarum*[3]
CL126	*L. parabuchneri*[3]	Not tested	No match
CL122	*L. parabuchneri*	Not tested	*L. parabuchneri*
CL112	No results	Not tested	*L. collinoides*[3]
CL113	Not tested	Not tested	*L. collinoides*[3]
CL118	Not tested	Not tested	*L. collinoides*[3]
CL100	*L. bifermentans*[3]	Not tested	*L. brevis*
CL101	*L. mali*[3]	Not tested	*L. casei casei*[3]
CL104	Not tested	Not tested	*L. brevis*
CL116	*L. bifermentans*[3]	Not tested	*L. collinoides*[3]
CL121	*L. helveticus*[3]	Not tested	*L. casei*
OMLA2	No results	Not tested	*L. mesenter* (OMLA7)
OMLA7	No results	Not tested	*L. mesenter*
OMLA9	Not tested	Not tested	*L. casei*
OML37	No results	Not tested	*L. mesenter*
OM	Not tested	Not tested	*L. casei*
RAK19	Not tested	*L. plantarum*	*L. plantarum*
RAK16	Not tested	*L. casei pseudo*	*L. plantarum*

[2] Repeatable results were not obtained
[3] Strains in parentheses matched test organism by ribotyping but did not match the database
[4] Low fatty acid concentration
[5] No identification made Closest species shown
[6] Strains not run for a variety of reasons
[7] Matched ATCC strain exactly
[8] All same pattern group
[9] Genus only identification Closest species shown

The orange juice cultures identified as *L mali* and *L bifermentans* in Table 22 were often only identified to genus at 24 hours. However, at 48 hours, the "closest species" designation given at 24 hours would become the identification. Often, if additional readings were taken, the identification tended to interchange between *L. mali* and *L bifermentans*, or the reverse. Therefore, it appeared that the unknown cultures were all the same organism, but it was not an organism that was providing a positive identification on the Biolog. The riboprinting results all presented the identical pattern for these strains which supported this theory. Because all the ribotyping patterns were the same, not all the cultures were run on the Biolog as well due to the cost of the tests.

Organisms that had no results were ones that had been run on the method, but test results were not obtained. In the case of Biolog, insufficient number of wells with a reaction was a common reason for no identification. In the case of the fatty acid profile, the quantity of cellular material was too low in many cases, to give a reading.

Figure 30 shows the results of the dairy isolate KR434 when compared to ATCC strain *Lactobacillus casei* ss *rhamnosus* (620). This is presented to illustrate the slight

[redacted] that were seen when analyzing the [redacted] isolates versus the ATCC strains. These slight differences can be noted by genetic analysis thus illustrating its power of discrimination and its application to epidemiological studies, but at the same time, demonstrating the need for careful analysis of results to recognize closely related species.

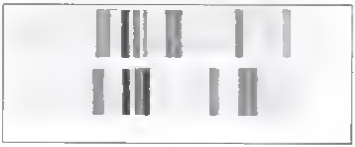


Figure 30. Dairy isolate KR454 (top) and *L. casei* (bottom)

Figures 31, 32, and 33 provide the dendrograms for the industrial strains in relation to the ATCC strains for the Biolog, fatty acid analysis, and ribotyping, respectively Samples in bold indicate those samples tested by all three methods.

In Figure 31, although many of the orange juice isolates gave the same identification using the Biolog, and clustered closely in this analysis, they did not cluster together as tightly as they did in the ribotyping dendrogram Also in the Biolog analysis, *Leuconostoc pseudomesenteroides* (429B) clustered more closely with members of the genus Pediococcus (712 and 4302) than with other *Leuconostoc* species

There were a number of differences between the dendrograms for the three methods The Biolog results clustered dairy isolate KR488 closely with *P. dextrinicus* while the ribotyping grouped this isolate with the lactococcal isolates It is important to note that in both cases, these strains were distinguished from members of the lactobacilli thus suggesting that they do belong in a different genus However, there was some overlap between the *Leuconostoc* and *Pediococcus* genera which indicates that members of these groups may be more closely related to

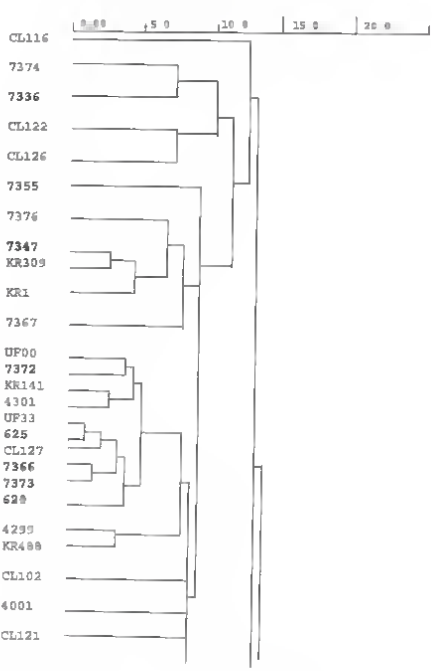


Figure 31 Results of Biolog analysis for all strains

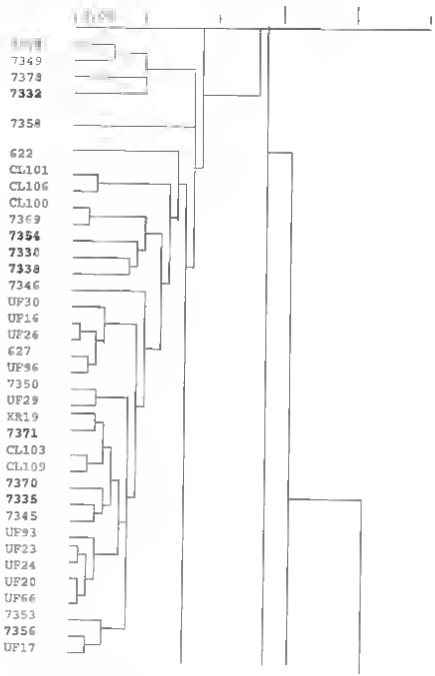


Figure 31--continued

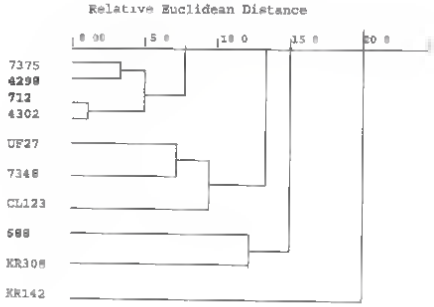


Figure 31--continued

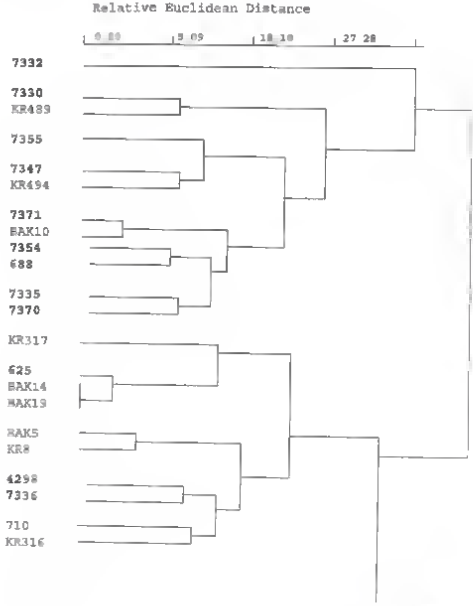


Figure 32 Results of fatty acid analysis for all strains

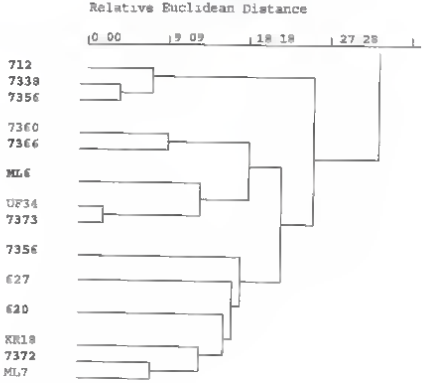


Figure 32 continued

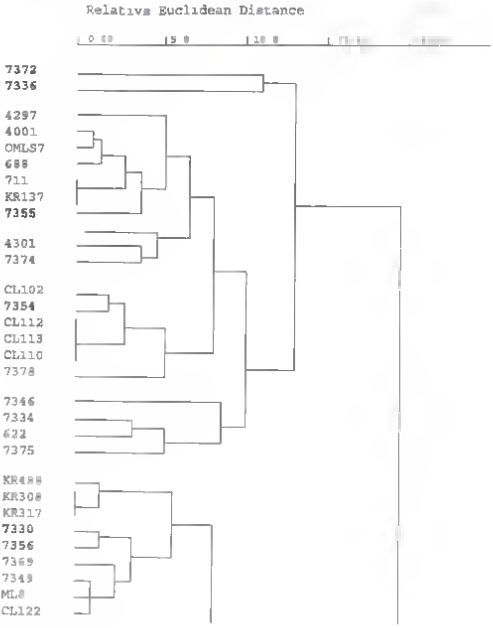


Figure 33 Ribotyping results for all strains

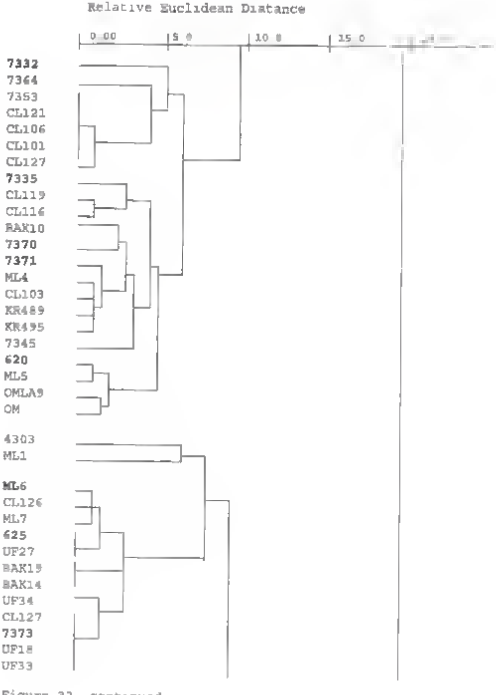


Figure 33--continued

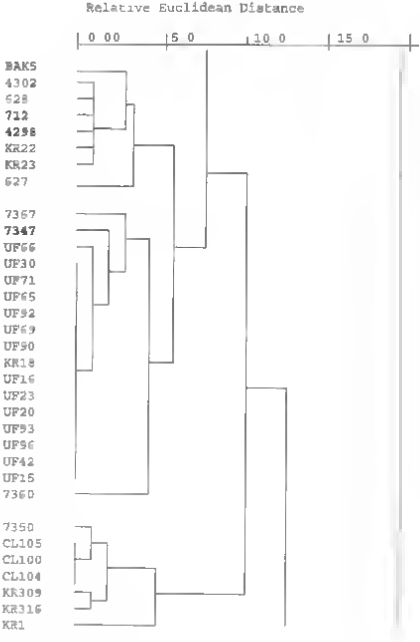


Figure 33 continued

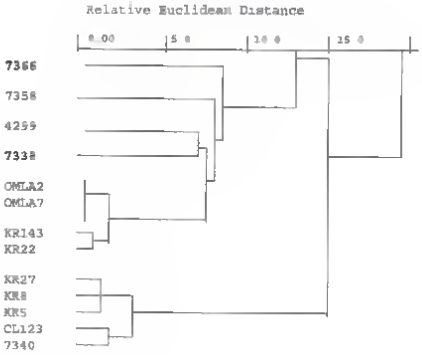


Figure 33—continued

species outside thier genus than species within

In the ribotype dendrogram, samples CL121, CL101, and CL116 clustered together however, by Biolog CL101 and CL116 clustered together, but not with CL121 There were similarities among these two tests also The *Lactobacillus plantarum* strains 625 and 7373 clustered in the same group which included CL127 and UF33. The fatty acid dendrogram did not cluster 625 and 7372 closely although the identification was correct. Fatty acid analysis clustered bakery samples BAK14 and BAK19 with *L plantarum* and close to BAK5 These same results were seen in the ribotype grouping

The method of identification did affect the results of the cluster analysis with some strains clustering closely when analyzed by one method and not closely when analyzed by another There was overlap between members of different genera and the most overlap occurred between members of the non-lactobacilli species Although there was overlap between clusters of genera, there did appear to be adequate differentiation between strains.

For the ribotyping, using the *EcoRI* restriction enzyme was successful for most organisms, but there were some large

groups of isolates with no distinction (specifically the orange juice isolates) This raised the question of whether these organisms were the same clonal strain or if the restriction enzyme could not differentiate between the strains The literature was reviewed to determine which other restriction enzymes might provide more discrimination

Other restriction enzymes have been tested for members of the lactic acid bacterial group and, in one instance, specifically for the *Leuconostoc* genus (81). *Hae* III fragments were used to identify members of *Leuconostoc* and the authors were able to distinguish between members of the genera *Leuconostoc* and *Lactobacillus*, but were not successful in epidemiological applications to the *Leuconostoc* species alone Similar results were generated in this research *Pediococcus* and *Leuconostoc* Riboprint band patterns generally had less bands than those of the *Lactobacillus* This meant that there was less opportunity for distinction See the appendix for the patterns Baloga and Harlander (7) reported the most discriminating enzyme for ribotyping *Listeria* species was *EcoRI* when compared to *HindIII* and *HaeIII* They also reported success with *EcoRI* for digestion of *Lactococcus lactis ss lactis* Rodtong and

Tannock (87) in an evaluation of ribotyping for *Lactobacillus*, screened 16 restriction endonucleases for their ability to digest These restriction enzymes included *ApaI*, *BamHI*, *BclI*, *BglII*, *ClaI* *EcoRI*, *HindIII*, *KpnI*, *KpnI*, *NaeI*, *PstI*, *PvuII* *SacI*, *SalI*, *SmaI* and *XhoI* *BclI*, *BglII*, *EcoRI*, and *HindIII* were selected for their study based on the screening results and it was concluded that *EcoRI* and *HindIII* were sufficient to characterize each of the strains tested They recommended the use of more than one enzyme to make an identification and recommended *EcoRI* and *HindIII* because they have very different recognition sequences, G/AATTC and A/AGCTT, respectively In another report using ribotyping as a method of distinguishing lactococci, four restriction enzymes were compared These included *PvuII* *EcoRI* and *HindIII* *HindIII* generated between four and 13 fragments and *EcoRI* produced less, four to six. These two enzymes were determined to be the most useful A report examining *Lactobacillus* strains in pigs used *EcoRI*, *HindIII*, and *PstI* to digest the DNA (55). In another report, authors evaluating strains of *Lactobacillus hilgardii* and *Lactobacillus brevis* used *EcoRI*, *BamHI*, *HindIII*, and *PstI* The probe they used was the 16S and 23S rRNA from

Escherichia coli They were successful in ribotyping all but two of the strains tested (59). Based on this information, selected strains of the orange juice isolates were tested using *BamHI* and *HindIII* The *BamHI* recognition sequence is G/GATCC The resulting patterns generated with *HindII* hed more bands than *EcoRI* (eleven to sixteen rather than eight), but the patterns were similar for the isolates tested (UF65, UF26, UF16, UF24, UF20, UF23). The *Lactobacillus* species tested as references had greater than 30 bands which was too many for meaningful analysis The *BamHI* showed similar results for the *Lactobacillus* strains, but did not digest the orange juice strains on three different attempts Control organisms did give patterns suggesting that these strains were resistant to being digested by *BamHI* Other restriction enzymes were not tested because the Biolog results indicated that these organisms were the same strain and the *EcoRI* had shown good discrimination with other samples prior to this set

Rapid Identification of Industrial Isolates

One challenge to creating a rapid method for identification of microorganisms is the creation of a

database that has the accuracy to identify organisms specifically enough to genus and species, but contains the flexibility to identify environmental isolates that are similar to the species in the database, but may differ slightly due to phenotypic or genotypic differences. As technologies advance, it is being discovered that there is even more bacterial diversity than previously considered (44). The results of these tests from environmental and industrial strains versus the ATCC strains supports this observation. The environmental isolates were much more difficult to identify by all three methods and often did not match the databases provided by the manufacturer or generated by this author using ATCC strains. For identification of unknown organisms, identification was facilitated when there was agreement among the different methods. Because the strains differed from the database, this author did not feel confident making a positive identification unless there was some correlation between methods. It is suggested that a combination of methods be used in these circumstances until a history of the contamination or normal flora of the foodstuff is developed. Once an organism is entered in the database, subsequent

identifications can be performed with higher confidence. This is demonstrated in the instance of the orange juice isolates. The same isolate was cultured on numerous occasions. After the organism was characterized using a combination of methods, ribotyping alone was used to provide the identification. Figure 34 shows the riboprint patterns from a number of these strains.

Fermentation Monitoring Results

There are a number of reasons to monitor fermentations that include gaining knowledge about the dynamics of a fermentation where the succession of microorganisms is not fully understood so that control may be exercised to influence the fermentation in a positive manner. The method must be simple and quick so that it can be performed routinely at the manufacturing facility conducting the fermentation. At present, most methods to identify bacteria require an isolation step to obtain a pure culture. Existing methods cannot identify unknown organisms that may coexist in a population. The three methods used in this research have the potential to be used to monitor mixed fermentations. In the case of Biolog, a mixed culture taken

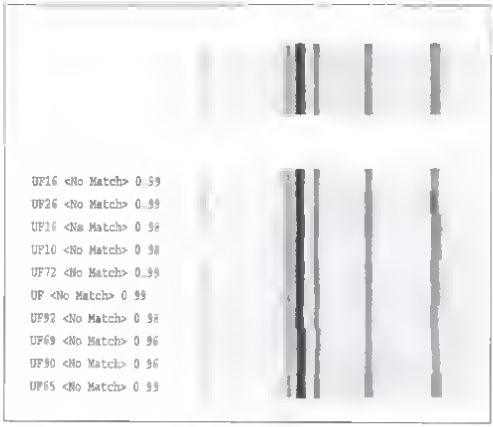


Figure 34 Ribotype patterns of orange juice strains the pattern at the top is the average of all patterns tested and is stored in the database The "No match" indicates that the samples did not match the database generated from ATCC strains

from a plate or pellet can be suspended in the Biolog broth and inoculated into a microwell plate. The resulting pattern would be specific for that mixture of organisms. The only problem is that in similar groups of organisms, reactions are similar. There may be only the difference of a few wells so that one organism may be able to "overshadow" the reaction of another. The other potential is that when organisms are very different, a great number of the wells will change color which limits the capabilities of distinction. Also, since use of the substrate is the key to the test, the age of the organism is a factor. In a mixed culture, the ages may differ within the population as well as the growth rates of different species. Organisms present in a fermentation nearing completion may react differently than organisms in a fresh fermentation. Also faster growing organisms will outgrow others that may still lend important characteristics to the fermentation. For these reasons, the Biolog was not chosen as the method to directly monitor the fermentations used in this research.

The fatty acid method of analysis also provides the potential for direct monitoring. Cultures in a broth can be centrifuged and the pellet used for fatty acid extraction.

(16). In fact, the entire raw material with culture can be used for a fatty acid extraction. If the principle components are selected, this method can be used to develop a profile of the fermentation. Unfortunately, the performance of the fatty acid analysis was not optimal in the pretests using the ATCC cultures. The method was relatively accurate in identifying to the genus level but was erratic in making a consistent identification to the species level. Also, the extraction method is time consuming, taking up to 5 hours with an additional few hours to run the gas chromatograph. For the analysis of one or two samples, the method is laborious. Finally, direct extractions from food samples require a traditional extraction procedure which is impractical at many manufacturing facilities. For the reasons discussed above the ribotyping method was selected to monitor the two fermentations.

Fermentation Results

The first steps in preparing to monitor the fermentations were to test the Riboprinter to determine whether mixed cultures could be distinguished as distinct patterns and to determine optimal cell density levels for

performing the ribotyping procedure from a liquid medium versus harvesting colonies from solid plates

The initial experiments used mixed cultures from solid plating media. The riboprint patterns were distinct and clear. When two different genera were mixed, the patterns did not overlap. Figure 35 shows the pattern for a mixed culture of *Lactobacillus plantarum* and *Leuconostoc paramesenteroides*. However, mixed cultures of *Lactobacillus* species resulted in many bands. Although they still created a distinct pattern that could be recognized as unique, it was not as clear as the mixed genus information. Figure 36 shows the results of *Lactobacillus casei* and *Lactobacillus sake*.

The next challenge was to determine when to harvest cells from a broth culture and the correct dose for the gel. Initial experiments were unsuccessful. No patterns were generated or the DNA was damaged. Figure 37 shows the gel results for one experiment. Lanes 1, 2, and 5 are all from solid media. Lanes 3, 4, and 6 were from broth cultures.

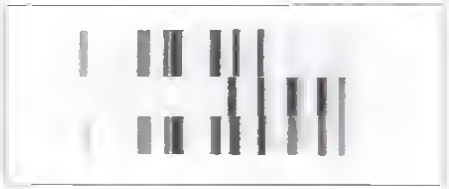


Figure 35 *L plantarum* (top), *L paramesenteroides* (middle) and both (bottom) from MRS agar

Figure 36 *L casei* and *L sake* from MRS agar

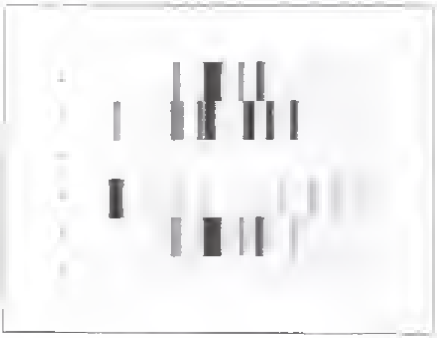


Figure 37 Initial broth ribotypes (lanes 3 4

Sausage Fermentation

The use of starters in the sausage industry enhances the safety of the product (9,84,95). Sausages inoculated with starter cultures tend to have lower pH values than non-starter, or naturally fermented, sausages (51). The production of lactic acid reduces the growth of gram-negative bacteria and the level of starter culture used increases the inhibitory effect (84).

Although the competitive microflora in ground beef is very high, the starter cultures selected are good competitors for a number of reasons. The cultures are quick growing and produce a variety of substances to reduce the competition. The glucose added is used as a substrate by the starter cultures and the nitrates are color stabilisers. *Lactobacillus plantarum* strains used in the meat industry as starter cultures reduce nitrate to nitrite via the enzyme nitrate reductase (95).

The fresh beef, inoculated with starter culture, resulted in a pure culture of that starter within 16 hours of the initiation of the fermentation. Figure 38 shows a Gram stain of the uninoculated beef and Figure 39 shows the beef after 20 hours of fermentation with starter *Pediococcus*

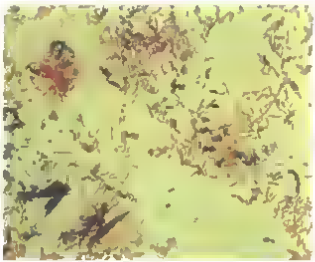


Figure 38 Normal flora of ground beef - Gram stain (x10,

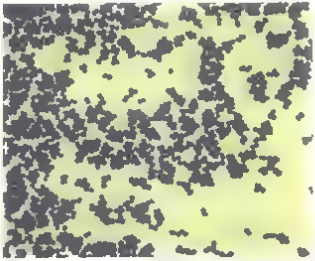


Figure 39 Microbial flora of sausage after 20 hours of fermentation Gram stain (x1000)

pentosaceus. It is apparent that the starter cultures have outgrown the normal flora present in the beef initially. The pH dropped to 4.8 in that time period as well, indicating the growth of the lactic acid bacteria and the production of acid. Other researchers report the same time for the pH change (17).

These results can be seen from the ribotyping results as well. Figure 40 shows ribotype patterns of the raw beef, starter culture and the sausage at 16 and 20 hours. The change in the flora can be seen as the starter culture begins to dominate the culture.

Sauerkraut Fermentation

Sauerkraut is produced from the normal flora of the cabbage. The salt and the anaerobic incubation conditions inhibit gram negative organisms which allows the growth of the lactic acid bacteria. *Leuconostoc mesenteroides* and *Lactobacillus plantarum* are the most common organisms found in sauerkraut cultures (51). The sauerkraut was more difficult to monitor in process than the sausage. When samples of cabbage were taken during the course of the fermentation, the oxygen introduced during sampling

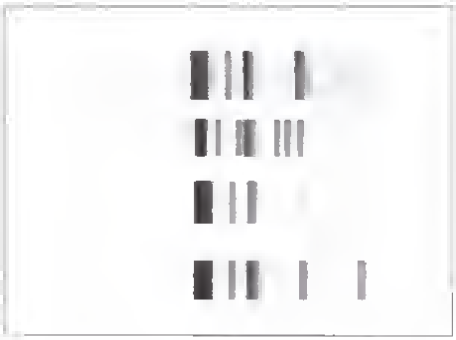


Figure 40 Ribotype patterns of sausage fermentation
The patterns from top to bottom: Starter culture (712) (top), uninoculated beef sausage flora after 16 hours of fermentation, sausage flora after 20 hours of fermentation

provided the opportunity for other organisms to grow and changed the flora of the fermentation so that instead of sauerkraut, the end product was rotten cabbage. Finally four separate fermentations were initiated from each batch of cabbage and after sampling, the portion was discarded. The normal flora of the cabbage was much lower than that of the ground beef.

Figure 41 shows the cabbage flora prior to fermentation. The pellets resulting from the stomaching and centrifuging step were small and all the cells were used for riboprinting. Figure 42 shows the cabbage after 3 days of fermentation. Although the culture was mixed, there was more bacterial activity overall. This is evident in the pictures.

Samples of the sauerkraut were taken weekly and a change in the flora could easily be seen in the ribotypes. The final pattern closely resembled that of the ATCC strain *L. mesenteroides*, although when cultured on solid plating media no growth was able to be obtained.

Figure 43 shows the ribotyping results of the sauerkraut at 0 time and week 1 through week 4. This experiment was considered a success because the cultures did

Figure 41 Raw cabbage microflora Gram stain (x1000)

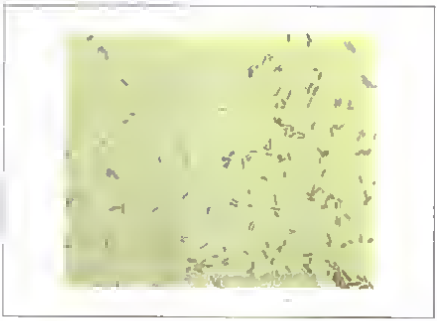


Figure 42 Cabbage after 3 days fermentation - Gram stain (x1000)

Count by [illegible] from 0 time (top) to [illegible]

not grow when cultured on plating media. Information was obtained about the cultures and the time of succession in the fermentation.

For both types of fermentations, the organisms used for the ribotyping were obtained by rinsing and centrifuging raw material and using this directly for riboprinting. The enzyme used was *Eco*RI and the probe was directed at the portion of *E. coli* DNA encoding for the 5, 16 and 23S portions of the ribosomal RNA. The results were obtained for analysis within 10 hours of sampling. Clear differences in the flora over time was easily observed. These patterns can be stored in a database and future fermentation patterns could be compared to ensure consistent and products

SUMMARY AND CONCLUSIONS

Strain Typing

Strain typing is as important to the food industry for diagnostic reasons as in the medical industry, only it is not widely applied due to the expense involved and the lack of convenient, reliable methods. Inoculation studies such as heat-killing determinations use strain typing to make strain specific comparisons of organisms recovered after a heat or chemical challenge to ensure that the process was adequate. For example, to study the efficacy of a heat process designed to kill *L. plantarum*, actual strains of *L. plantarum* may be inoculated into the product and then processed. The final product would be incubated and plated after the heat challenge. If organisms are recovered they can be typed to the strain level to determine if they were the same organism that was inoculated or an environmental contaminant. Also strain typing can be used to trace contaminating raw materials or equipment. The Campden Food

[illegible] Research Association has been using Riboprint [illegible] patterns

> to pinpoint the sources and track the spread of contamination within factories, identify problem suppliers and monitor changes in the microbial composition of foods over time This has contributed to a reduction in product development times, identification and control of quality loss and spoilage populations and characterization of beneficial fermentative starter cultures" (67:15)

It is the desire of this author that this and other research will facilitate the introduction of more sophisticated methods of strain typing to food research institutions and manufacturing facilities so that they become accessible tools to be used more routinely in a field where traditional microbiology still dominates

Method Comparison

The Biolog is useful in screening microorganisms because a large number of biochemical tests can be performed simultaneously There are some areas of concern Slow growing organisms are still difficult to analyze The biomass formed by slow growers is too low to degrade sufficient substrate amounts and the test results may be unclear Also, some characteristics of organisms are

unstable, varying with the preculture media or genetic variants (i.e. plasmids).

The analysis of cellular fatty acids also requires a large cell quantity of pure culture for the analysis of lactic acid bacteria. Growth conditions such as age of culture, growth media, growth temperature, and atmospheric conditions, are critical. Slow growing organisms are also difficult to analyze due to the quantity of cells required and the tightly controlled incubation times. However, the shelf life of the reagents is not a problem and after extraction, the system can run unsupervised (104). After the initial investment of equipment, the cost of the disposables is relatively inexpensive, but there is intensive labor required (92).

The ribotyping technique does not require the large amount of cellular material that is needed for the other tests and the automated system overcomes the need for highly technical personnel to perform the analysis. The cost of the test is high both in the initial equipment investment and disposables, but labor is minimal and the type of information gained is powerful. The patterns generated for the genera *Leuconostoc* and *Pediococcus* were not as diverse

those generated for the *Lactobacillus* group and many of the environmental isolates were grouped into the same Riboprint pattern. The use of different restriction enzymes may be necessary to increase the specificity of the test. Over or underdosing of the gel will affect the number of bands and older cultures may experience some endonuclease activity, resulting in differing band patterns. Incomplete lysis was a common problem with the lactic acid bacteria although recent developments by the manufacturer have addressed this issue. The specific bands are not weighted in the computer which makes identification of unknowns more difficult.

Identification of Environmental Isolates

Since environmental isolates often vary both phenotypically and genotypically from the library of strains located in the culture collection, it is difficult to make an accurate determination of a species' taxonomy using only one method. The lactic acid bacteria, in particular, are largely uncharacterized; therefore, current methods must be adapted or combined to make taxonomic judgments. Other researchers recommend combinations of methods to identify

other bacterial genera (5) For the initial characterization of a culture, this author recommends a combination of the three methods to gain the best information and confidence in the results. In a study comparing the Biolog to the MIDI system for fatty acid analysis, the Biolog correctly identified 69/81 non-fermenting Gram negative organisms to the genus level and the MIDI system correctly identified 70/81 to the genus level. Speciation was less successful and tended to be difficult for species that are difficult using conventional means. These authors also concluded that neither system should be used as a stand alone method for identification (41)

Some authors believe that "the goal of DNA probe technology is to eliminate routine cultures, whether they be bacterial, viral or fungal" and that genetic sequencing techniques will become a standard tool for characterizing unknown bacteria (57,117) Molecular analysis is by far the most popular new approach to classification and it has tended to overshadow the more classical means of taxonomy, however this author believes that a combination of methods may be the most representative means of classifying

microorganisms. Direct genomic similarities do not always translate into phenotypically related organisms. This area of study is one that brings great challenge, explanation of the relationship between molecular and phenotypic variances.

The Future of Microbial Taxonomy

The integration of the data obtained by one or more of the many emerging methods of analysis creates a new challenge for the microbiologist of today. Traditional classification methods may be a foundation on which to compare the newer, more technologically advanced systems. Furthermore, genetic comparisons are extremely useful in epidemiological studies (18,50).

Once an organism has been determined to be of importance, a polymerase chain reaction method (PCR) can be developed to quickly determine if it is present. This has been tested with success in some vegetable fermentations for species in the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus* (15). Already, methods exist that challenge ribotyping. Many authors have demonstrated a greater ability to distinguish between strains using pulsed-field gel electrophoresis when compared to ribotyping and

traditional plasmid profiling (94) Another emerging methodology showing great promise is Random Amplified Polymorphic DNA (RAPD) RAPD incorporates PCR technology, but uses short primers to amplify random fragments that can be analyzed via gel electrophoresis This tool allows the detection of polymorphisms within closely related bacteria (14,116) It may be a further discriminatory tool to be applied after ribosomal patterns reveal genetic relatedness This has also been used as a strain specific method to identify members of the lactic acid bacterial group, specifically *Lactococcus lactis ss cremoris* and *lactis* (36)

Data Analysis

The use of automated systems coupled with the analytical capacity of computer systems has impacted the world of microbiology to an enormous capacity For example individual comparisons of each of the Biolog 96 wells would be extremely time consuming The software available for taxonomic comparison of bacterial isolates allows for numerical taxonomy and identifications based on probabilities In addition, the introduction of new molecular methods has caused many of the traditional

classifications to come into question. The lactic acid bacterial group is particularly inclined to be questioned because of its rather vague historical classifications to begin with. The ability to store this data for further comparisons has led to the development of huge strain databases. This is critical as microbiology is still a study dealing with live organisms that have the ability to change from day to day. These biomolecular databases are proliferating and this is changing the face of research (72). Bioinformaticians are struggling to make sense of the growing inventory of genetically encoded information (22). The way in which researchers use information is changing and the challenge of integrating the information is one that is becoming a field of study in itself (72). This data is growing, in part, due to the availability of data and the ability of people to share this data.

The internet is an extremely valuable tool because it provides the ability to disseminate information quickly and widely. The attractiveness of systems that are computerized is that the data generated can be directly distributed through the internet. Articles are being published that refer to the internet and its databases such as GenBank and

the Ribosomal Database Project (38) In the case of the Dupont ribotyping system, actual DNA "fingerprints" can be directly compared electronically

Bacterial Diversity

As methods of bacterial identification improve, the diversity of microorganisms is more apparent and it is apparent that actual bacterial populations within an ecosystem greatly exceeds that which is culturable by today's methods The causes may be that the bacteria are not culturable due to environmental stresses or that conventional methods are not applicable for these strains (3) For whatever the reason, the application of molecular methods are essential

The field of microbiology continues to incorporate ecology or the study of mixed cultures, pure culture analysis and characterization; metabolic studies, analysis of cell extracts for enzymatic purposes, and, finally, molecular biology These areas are all linked together and the greatest information is obtained when the microbiologist has exposure to the tools that will let he or she best characterize and study the situation of interest (13,80)

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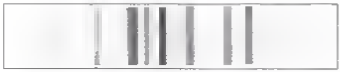
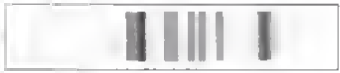
APPENDIX

This appendix contains the actual riboprints in greyscale form for the ATCC strains tested on the Riboprinter

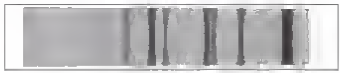
Lactobacillus alimentarius ATCC 29643



Lactobacillus amylovorus ATCC 33620

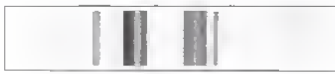


animalis ATCC 35046



Lactobacillus bifermentans ATCC 3541

Lactobacillus brevis ATCC 27305



Lactobacillus *brevis* ATCC 14869

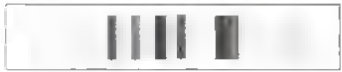
Lactobacillus casei ss casei ATCC 393

Lactobacillus casei ss rhamnosus ATCC 7469

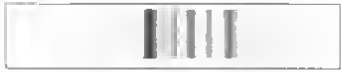
Lactobacillus collinoides ATCC 27612

Lactobacillus confusus ATCC 10881

Lactobacillus coryniformis ATCC 25603



Lactobacillus coryniformis ss *torquens* ATCC 25600

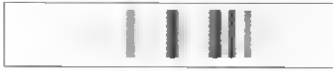


Lactobacillus curvatus ATCC 25601

Lactobacillus delbrueckii ATCC 9649

delbrueckii ATCC 8000

Lactobacillus farciminis ATCC 29644



Lactobacillus fermentum ATCC 14931

Lactobacillus fructosus ATCC 33162

Lactoba[illegible] ATCC 35410

Lactobacillus hilgardii ATCC 8290

Lactobacillus malefermentans ATCC 49373

Lactobacillus murinus ATCC 35020

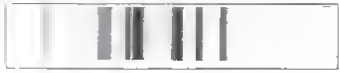
parabuchneri ATCC 49

Lactobacillus paracasei ATCC 25599

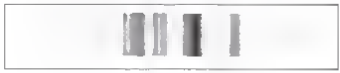
Lactobacillus paracasei ATCC 25302

Lactobacillus pentosus ATCC 8041

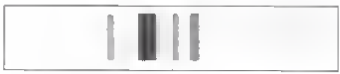
Lactobacillus plantarum ATCC 14917



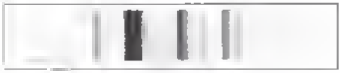
Lactobacillus plantarum ATCC 8014



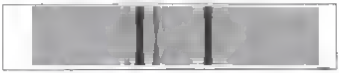
Lactobacillus sake ATCC 15521



Pediococcus acidilactici ATCC 33314



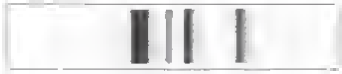
Pediococcus acidilactici ATCC 8081



Pediococcus acidilactici ATCC 8042

Pediococcus damnosus ATCC 25358

Pediococcus dextrinicus ATCC 33087



Pediococcus pentosaceus ATCC 33316

Leuconostoc citreum ATCC 49370

Leuconostoc mesenteroides ATCC 19255

Leuconostoc mesenteroides ATCC 19254

[illegible] *mesenteroides* [illegible]

Leuconostoc paramesenteroides ATCC 33313

Leuconostoc pseudomesenteroides ATCC 49371

Enterococcus faecium

Enterococcus faecalis

Lactococcus garvieae ATCC 43921

BIOGRAPHICAL SKETCH

Susan Matilda Freund was born in Milton, Florida, to Harman Clifford and Susan Bridgett Freund. She has one brother, David Thomas Murray. Her father was a US Naval pilot and she moved frequently during her childhood. She graduated from high school in Ankara, Turkey.

Matilda first attended The College of Wooster in Ohio before transferring to the University of Florida in Gainesville. She received a Bachelor of Science in microbiology and cell science in 1982 and, in 1987, she obtained a Master of Science in food science and human nutrition with a minor in environmental engineering.

She has worked as a research microbiologist in a clinical laboratory at the University of Florida's College of Veterinary Medicine and as a consultant and laboratory inspector for the State of Florida Water Certification Program. Matilda began working for Kraft Foods in 1990 as a food microbiologist and has held positions in research, quality, product development, and operations.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

Cheng-i Wei, Chair
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Mickey E Parish
Associate Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Mark L Tamplin
Associate Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

James F Preston
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard A. Chordash
Director, Food Science
Kraft Foods

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Hugh L. Trenk
Associate Director,
Biological Sciences
Kraft Foods

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1996

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School